# An Investigation into Fungal Metabolism of Halogenated and Related Steroids

by

Everton M. Thomas, B.Sc. (Hons.) (Brock)

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## ABSTRACT

Fungal metabolism of halogenated and related steroids was investigated. The fungi Aspergillus niger ATCC 9142, Curvularia lunata NRRL 2380 and Rhizopus stolonifer ATCC 6227b were studied in this regard.

21-Fluoro-, 21-chloro, 21-bromo- and 21-methyl-pregn-4-ene-3,20-diones were prepared and incubated with A. niger (a C-21-hydroxylator) in order to observe the effect of the C-21 substituent on the metabolism of these substrates. In all four cases, the C-21 substituent prevented any significant metabolism of these substrates.

11β-Fluoropregn-4-ene-3,20-dione was prepared and incubated with C. lunata (an 11β-hydroxylator) and R. stolonifer (an 11α-hydroxylator).

With C. lunata, the 11-fluoro- substituent prevent hydroxylation at the 11 position, but diverted it to a site remote from the fluorine atom. In contrast, with R. stolonifer the 11β-fluoro- substituent, although slowing the apparent rate of hydroxylation, did not prevent its occurrence at the 11α- position.

11β-Hydroxypregn-4-ene-3,20-dione was also incubated with R. stolonifer. The 11β-hydroxy- group did not appear to have any significant effect on hydroxylation at the 11α- position.

The incubation of a substrate, unsaturated at a favoured site of hydroxylation with Rhizopus arrhizus ATCC 11145 provided a complex mixture

of products; among them were both the α and β epoxides. The formation of these products is rationalized as arising because of the lack of regio- and stereospecificity of the hydroxylase enzyme(s) involved.

ACKNOWLEDGEMENTS

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Dedicated to

Gladys

who made it all possible

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# INTRODUCTION

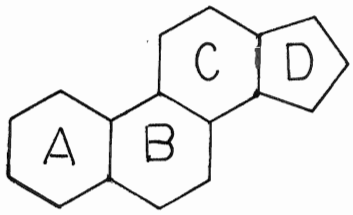
## The adrenocortical hormones

The steroids are members of a large class of compounds which possess the perhydro-1,2-cyclopentenophenanthracene ring system [1] as a common feature. The steroids include a wide variety of naturally occurring and synthetic substances such as sterols, bile acids, adrenocortical hormones, sex hormones, various contraceptive drugs, cardiac glycosides, insect molting hormones and antibiotics.

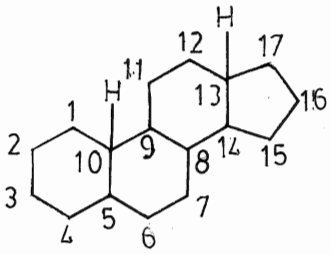
The two carbon acetate is the biosynthetic precursor of the steroids and the familiar sterol, cholesterol [8] is the prime source of all steroids in the human organism. Cholesterol is biosynthesized through a complex series of 25 reactions involving the participation of 18 acetate units.

Steroid nomenclature is based upon the nature of the hydrocarbon framework from which the steroid is derived. Individual compounds are named systematically as derivatives of steroidal hydrocarbons. The more important of these are formulated and numbered below (Fig. 1). The carbon atoms are numbered in a predetermined sequence beginning with ring A. The Greek letter Δ indicates a double bond as does the suffix -ene. Substituents attached to the ring system from above are designated β (solid line), those attached from below are designated α (dotted line). This study deals only with steroids having the androstane [4] and pregnane [5] skeleton.

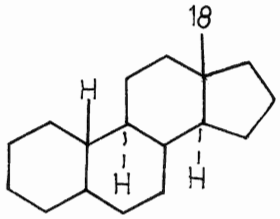
Figure 1. Common steroid skeleton



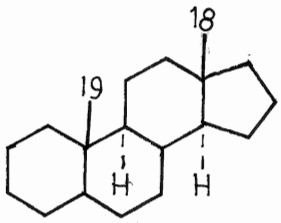

[1]



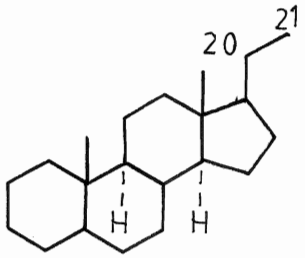

gonane [2]



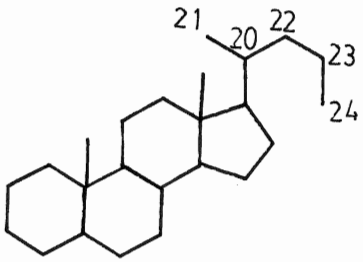

estrane [3]



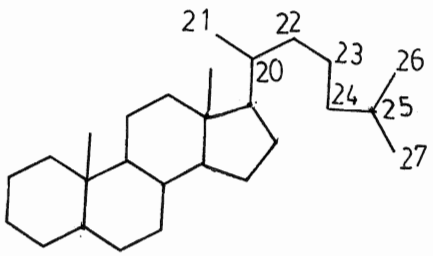

androstane [4]




pregnane [5]




cholane [6]

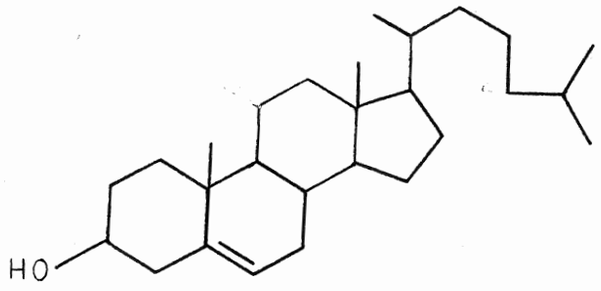



cholestane [7]

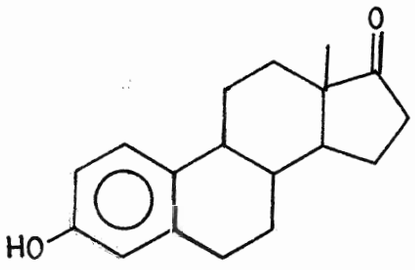
In the next few pages, a brief introduction is given regarding the discovery of the glucocorticoid hormones and their synthetic analogues. Although the glucocorticoid hormones are not directly related to the present study, two aspects in their development, namely the discovery of the widespread microbiological transformation of steroids and the observation that the introduction of a halogen in the steroid nucleus had a dramatic effect on the physiological activity of these new analogues, are important and as such the following brief account should put this study in context.

In 1927, Rogoff and Stewart[1] extracted fresh dog adrenal glands and found that the clear extract could be injected intravenously into adrenalectomized dogs (see below) without any evident ill effect. (Human adrenal glands are small organs weighing about 6 g each located near the upper end of each kidney.) Rogoff and Stewart also observed that double adrenalectomized dogs (dogs in which both adrenals had been removed) survived an average of 7-10 days after surgery. They also observed that of 30 adrenalectomized dogs injected with the adrenal extract, 6 survived 18-78 days and the rest compared favorably with the controls. Thus it was concluded that the adrenal extract in some unknown way prolonged the life of the animals in the absence of their adrenals.

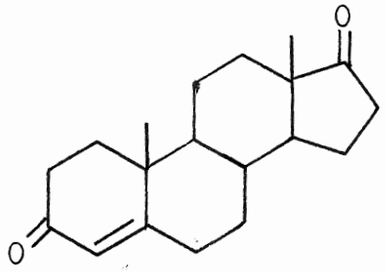
Over the next decade, several groups[2,3] tried successfully to isolate the individual components of the adrenal extract and by 1943, no less than 28 steroids had been isolated.[4] The list includes estrone [9], androstenedione [10], progesterone [11], a number of compounds having no known biological activity and six $\Delta^4$-3-ketosteroids soon



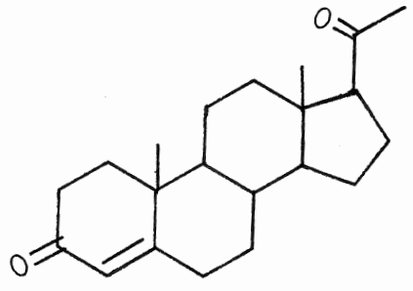

cholesterol [8]




estrone [9]




androst-4-ene-3,17-dione [10]




progesterone [11]

recognized as having one or another type of activity specific to the adrenal function. The six biologically active compounds having the $\Delta^4$-3-ketone function are all progesterone derivatives and all contain the α ketol side chain differing only in the nature of the function at C11 and C17 (Fig. 2).

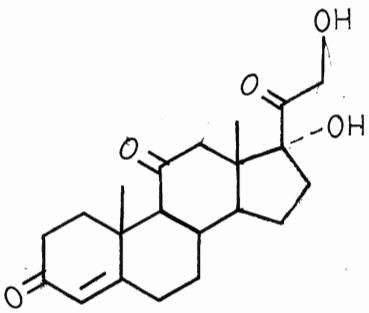
In the decade that followed the discovery of the above compounds, no very significant use was indicated. Investigation of the biological action of individual compounds disclosed that they possess, in varying degrees, activities of several types.

In tests measuring ability to increase survival of adrenalectomized animals, the highly oxygenated cortisone [12] and cortisol [13] showed only low order of activity, whereas cortexone [17], the least oxygenated was very potent.[5]

A significant function of some of the compounds listed in Figure 2 is their ability to promote deposition of glycogen in the liver. This liver glycogen or glucocorticoid activity is highest for cortisol [13] and cortisone [12], whereas cortexone [17] is inactive. Another manifestation of activity of the steroids in Figure 2 is their ability to control the balance of body fluids. A substance is said to have electrolytic metabolic activity or mineralocorticoid activity if it promotes increased retention of $Na^+$, $Cl^-$ and water and excretion of $K^+$. Such manifestation leads to edema which is usually objectionable. Cortexone [17] has the highest activity of this type.

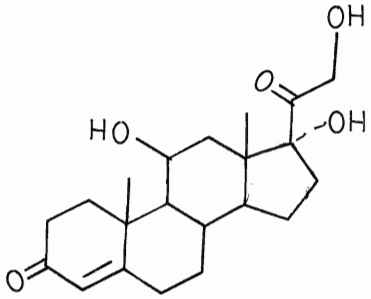
The varied manifestation of biological activity did not suggest any further therapeutic application of the active compounds in Figure 2 and the substances which appeared most interesting because of their high

Figure 2. Glucocortical hormones



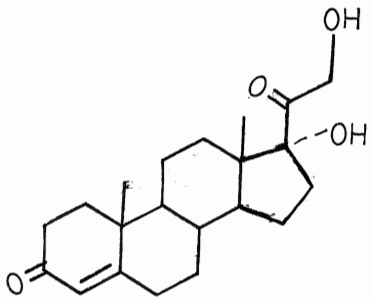
cortisone [12]

17β,21-dihydroxypregn-4-ene-3,11,20-trione



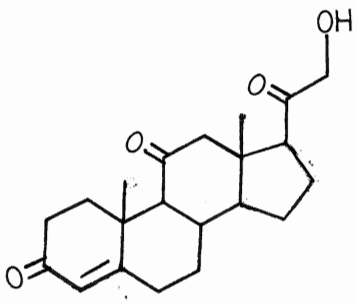
cortisol [13]

11β,17α,21-trihydroxypregn-4-ene-3,20-dione



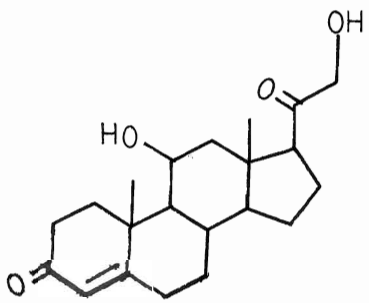
cortexolone [14]

17α,21-dihydroxypregn-4-ene-3,20-dione



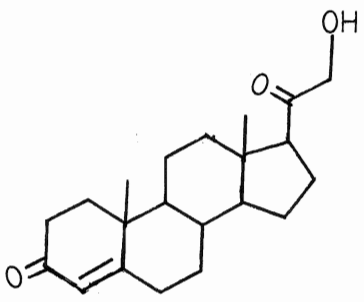
11-dehydrocorticosterone [15]

21-hydroxypregn-4-ene-3,11,20-trione



corticosterone [16]

11β,21-dihydroxypregn-4-ene-3,20-dione



cortexone [17]

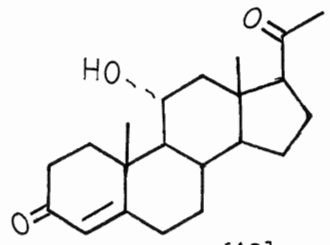
21-hydroxypregn-4-ene-3,20-dione

carbohydrate activity were not available by isolation in amounts adequate for exploratory clinical experimentation. The striking application to therapy that eventually ensued developed as the result of extensive chemical investigation starting with the elucidation of the structures. By the late 1940's, the structures shown in Figure 2 had all been elucidated and it was established that cortisol [13] was the most important adrenocortical hormone.[6]

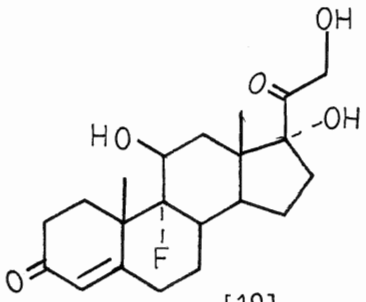
In 1949, clinical tests performed by Hench and Kendall[7a] revealed that cortisone [12] was a powerful drug in the treatment of rheumatoid arthritis. This discovery provided a strong incentive for further research directed towards the efficient and inexpensive preparation of this hormone. One major problem encountered in attempts to prepare cortisone from the available steroids was how to efficiently introduce the biologically necessary oxygen at carbon 11. Although many chemical approaches were tried, they were generally long and inefficient.

The important discovery by an Upjohn group headed by D. H. Peterson[8] that the soil fungus Rhizopus arrhizus introduces an hydroxy group at carbon 11 of progesterone [11] in 50% yield provided the breakthrough needed to accomplish the efficient synthesis of cortisone [12]. Shortly thereafter, many other organisms were discovered which were able to introduce the C-11 hydroxy group more efficiently. The fungus Rhizopus nigrans produces 11α-hydroxyprogesterone [18] from progesterone [11] in 80-90 percent yield.[8,9]

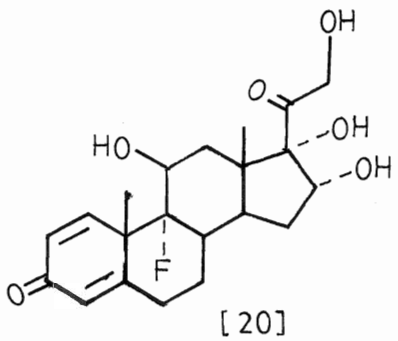
It soon became apparent that treatment of rheumatoid arthritis with cortisone [12] only alleviates rather than cures the disease. Therefore, it was necessary for treatment to continue over protracted time periods.




[18]




[19]




[20]

After cortisone [12] had been used for some time in the treatment of rheumatoid arthritis, undesireable side effects became evident with increasing frequency, particularly on long term usage.

The cortical hormones have a wide spectrum of activity and the small concentrations normally maintained by the adrenal cortex regulates a variety of metabolic processes that are essential to life. Therefore when cortisone [12] and cortisol [13] are applied at dosage levels necessary to suppress the symptoms of rheumatoid arthritis, some of the metabolic effects may reach abnormal proportions. Therefore, side effects such as excessive sodium retention and potassium excretion, negative nitrogen balance, increased gastric activity, edema and psychosis are exaggerated manifestations of the normal metabolic functions of the hormones.[7b]

The hope thus arose that some modification of structure might make possible a separation of the undesireable side effects from the anti-rheumatic activity or at least produce a shift in the relative intensities of the two types of action. Many attempts were made at modifying the structure of cortisol, but the resulting products generally had lower anti-rheumatic activity than cortisol itself.

Another breakthrough came about when it was accidentally discovered that the introduction of a fluorine atom at the C9 position of cortisol increased the potency of this new analogue ten-fold, making this new analogue, 9α-fluorocortisol [19] effective at one-tenth the dosage of cortisol.[10] This new analogue, however, was not free of harmful side effects since it resulted in an increased mineralocorticoid activity,

that is the causation of the retention of salt and water. This property is objectionable since it leads to edema.

The introduction of the other halogens, chlorine, bromine and iodine at the 9α-position of cortisol also provided new analogues with anti-inflammatory activity although their activities were lower than that of cortisol.

After the above discovery, much effort was expended by many investigators trying to introduce the halogens at other positions of the steroid nucleus. The introduction of a fluorine atom at the 12α position of cortisol was reported to have the same effect on glucocorticoid activity as introduction at the 9α-position.[11] Surprisingly enough, introduction of a chlorine at the same position shows no appreciable activity in either the glucocorticoid or the sodium retention tests.[12] The introduction of a fluorine atom at the 6α position of cortisol increased the anti-inflammatory activity by a factor of 10-12.[13,14] Also, the replacement of the 21-hydroxy group of cortisol by a fluorine atom resulted in an increase of 3-5 times the glucocorticoid activity.[15] The same substitution in progesterone [11] increased the progestational activity by a factor of 2-4[16] Subsequently, other functional groups were introduced along with the halogens to further separate the undesireable side effects from the desired ones. A good example is triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxypregn-1,4-diene-3,20-dione [20]) which has the original cortisol structure and has been modified at 3 positions; the introduction of the 16α-hydroxy group decreased the salt retaining properties that usually accompany the introduction of an halogen atom. At the same time, the introduction of the C1-C2 double

bond (accomplished microbiologically) also increased the anti-inflammatory activity with a concommitant decrease in the undesireable salt-retaining side effects.[17]

The hydroxylation of steroids

It was mentioned earlier that the discovery of a strain of the Rhizopus species that could hydroxylate the steroid nucleus at carbon-11 helped resolve the problem of preparing cortisol and cortisone economically from abundantly available starting materials. After the above discovery, much time was invested in screening micro-organisms that were able to functionalize the steroid nucleus at various positions and in various ways. Today, micro-organisms are known which can transform steroids in numerous ways. The many types of transformation known are listed below.[18a]

1. OXIDATIONS
   - hydroxylation
   - dehydrogenation
   - epoxidation
   - oxidation of alcohols to ketones and aldehydes
   - oxidation of ketones to esters and lactones
   - oxidation of sulfides to sulfoxides
   - oxidation of amines to ketones
2. REDUCTIONS
   - reduction of ketones, aldehydes and acids to alcohols
   - reduction of double bonds
   - reduction of bromide

3. ESTERIFICATION, AMIDE FORMATION AND HYDROLYSIS

   hydrolysis of esters to steroidal alcohols

   hydrolysis of oxides to alcohols

   hydrolysis of acetals to steroidal alcohols

   N-acylation of amines

4. ISOMERIZATION

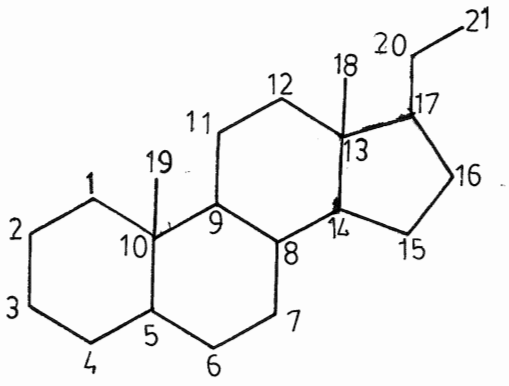
5. RESOLUTION OF RACEMIC MIXTURES

The hydroxylation and dehydrogenation reactions are currently the most important commercially. This is, of course, due to their role in the partial synthesis of the adrenocortical hormones and their analogues. The hydroxylation reaction is unique among the transformations listed above inasmuch as no particular substrate is required except saturation at the site to be hydroxylated.

The microbial enzymes display a formidable, widespread and remarkable ability to hydroxylate steroids. That the reactions are indeed enzymic has been proved in several cases by the isolation of the crystalline enzymes from the microbial species and by subsequent transformation of the steroid *in vitro* using the crystalline enzyme and an added reagent. The resulting transformations were identical with those obtained employing the intact microbial systems with the same substrate.[19] At present, reports have been made claiming the observation of microbial hydroxylation at almost all positions of C21 steroids as shown in Figure 3.[18b,35]

However despite the vast number of papers that have been published on microbial hydroxylation of steroids, and despite their continuing

Figure 3. Positions of normal C-21 steroids reported to be hydroxylated by micro-organisms[18a,35]




| | | |
|---|---|---|
| 1α | 8β | 17α |
| 1β | 9α | 18 |
| 2α | 11α | 19 |
| 2β | 11β | 21 |
| 3β | 12α | |
| 4β | 12β | |
| 5β | 14α | |
| 6α | 15α | |
| 6β | 15β | |
| 7α | 16α | |
| 7β | 16β | |

industrial importance, many aspects of steroidal hydroxylation remain obscure. The enzyme mechanisms of steroid hydroxylation have been studied intensively in various mammalian systems for many years. Later investigation on microbial conversion of these compounds has added to the elucidation of steroid metabolism and its enzymic basis. However, much work remains to be done before the detailed mechanism of steroidal hydroxylation is fully understood.

The pioneering work on the mechanism of steroid hydroxylation was carried out by Hayano and his collaborators.[20] They used beef adrenal homogenates to 11β-hydroxylate a variety of steroids which were saturated at carbon-11. On the basis of tracer studies, it was established that the oxygen of the hydroxyl group originated from molecular oxygen and not from water or any other oxygen-containing compound present in the medium; thus, when 17α,21-dihydroxy-pregn-4-ene-3,20-dione [14] was incubated with beef adrenal homogenate and $H_2{}^{18}O$, the product cortisol [13] contained only normal amounts of $^{18}O$. When, on the other hand, 21-hydroxypregn-4-ene-3,20-dione [17] was incubated in $^{18}O_2$ atmosphere with the same enzyme preparation, all the newly incorporated isotope was found to reside in the newly introduced 11β-hydroxy group of 11β,21-dihydroxypregn-4-ene-3,20-dione [16].

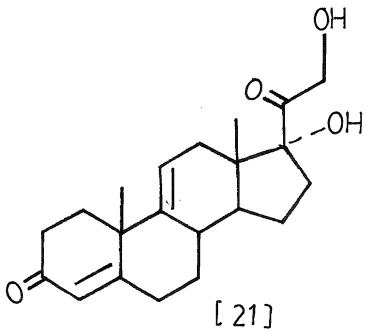
It was then easily shown that the source of the oxygen of the hydroxyl group was the same for microbial hydroxylation of steroids.[21,22] It was also shown that the 9(11) unsaturated analogues remained unaffected when incubated with beef adrenal homogenate, thus making them unlikely intermediates in the 11β-hydroxylation of [14] and [17]. Similarly,

neither 17α,21-dihydroxypregn-4,9(11)-diene-3,20-dione [21] nor pregn-4,16-diene-3,20-dione [22] was transformed when incubated with micro-organisms which hydroxylate the corresponding steroids at the 11α and 16α positions.

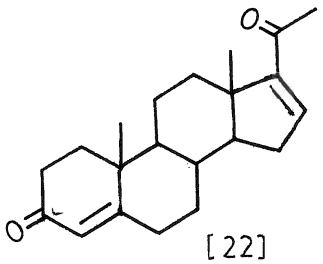
Still more evidence was obtained through the findings of Bloom and Shull[23] that micro-organisms convert double bonds into oxides rather than alcohols. They fermented 17α,21-dihydroxypregn-4,9(11)-diene-3,20-dione [21) with the 11β-hydroxylating fungus Curvularia lunata and obtained the 9β,11β-oxido-17α,21-dihydroxypregn-4-ene-3,20-dione [23]. Similarly, 17α,21-dihydroxypregn-4,14-diene-3,20-dione [24] was converted by the same fungus into 14α,15α-oxido-17α,21-dihydroxypregn-4-ene-3,20-dione [25].

As a result of the above findings, Bloom and Shull postulated that a micro-organism that is capable of introducing an axial hydroxyl group at $C_n$ is also capable of converting a dehydro- substrate into an oxide also axial at $C_n$. This deduction is consistent with the previous finding that incubation of [21] with the equatorial 11α-hydroxylating fungus Aspergillus nidulans failed to produce [26].

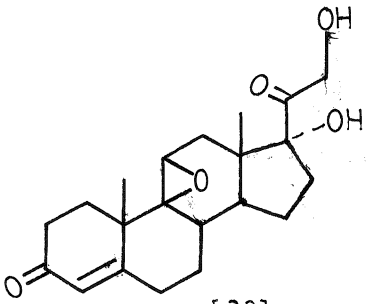
The use of tritiated and deuterated substrates provided more information regarding the stereochemistry of the enzymic hydroxylation. Pregnane-3,20-dione-11α$^3$H,12α$^3$H was incubated with Rhizopus nigricans which provided an 11α-hydroxy-derivative which showed a loss of 69% of the



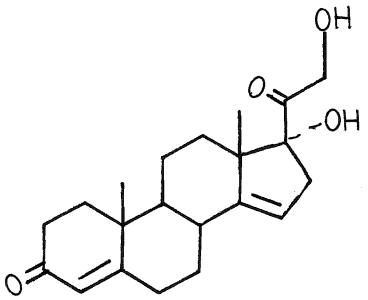

[21]




[22]




[23]

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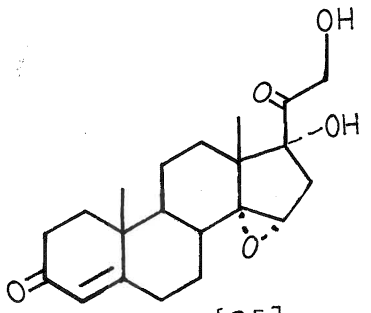
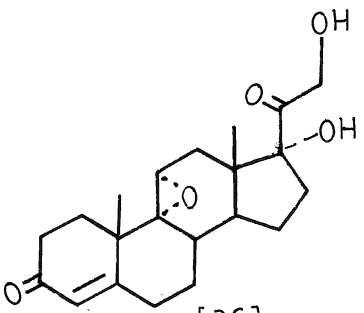



[25]




[26]

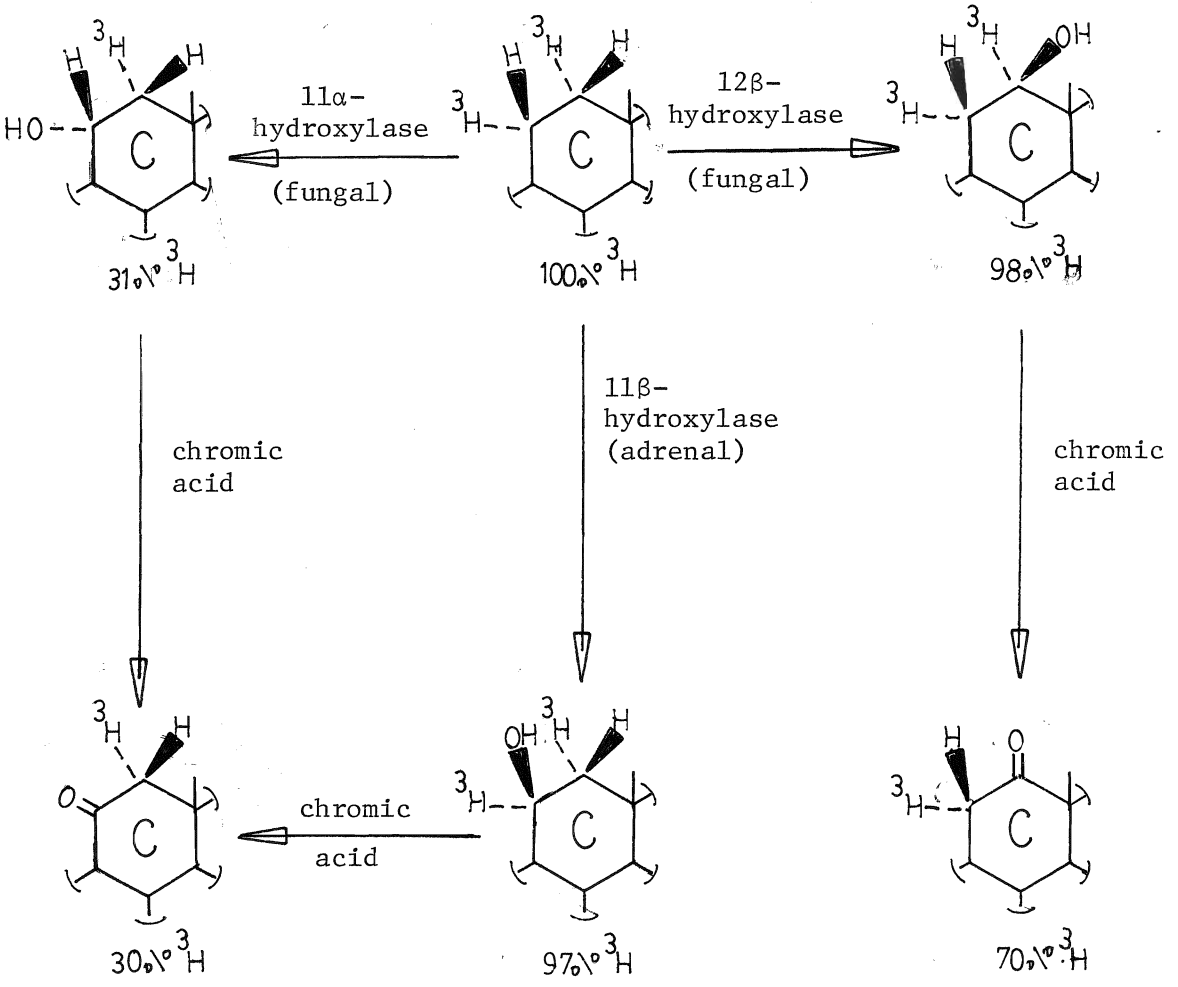
total count of the substrate. After mild oxidation of this product, essentially no change in the counts were observed. On the other hand, when progesterone-11α$^3$H,12α$^3$H was perfused through surviving adrenals, it yielded two 11β-hydroxy analogues, namely cortisol [13] and corticosterone [16]. No loss of the total substrate count was observed at this step, but there was a 70% decrease on subsequent oxidation with chromic acid[24,25] (Fig. 4).

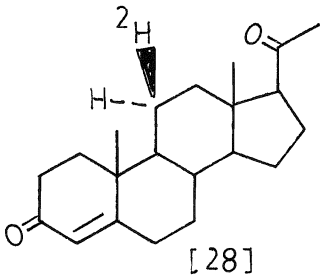
In a subsequent study[25] progesterone-11α$^3$H,12α$^3$H was incubated with Calonectria decora, an organism which dihydroxylates certain steroids at the 12β and 15α positions. The isolated dihydroxy metabolite showed essentially no loss in activity in comparison with the original count of the substrate. Mild oxidation by chromic acid of the metabolite to the corresponding 11,15-dioxo analogue resulted in the loss of 30% of the original count (see Fig. 4).

The above findings demonstrated that the stereochemistry of the carbon atom which is hydroxylated is always preserved. The newly formed hydroxyl group has the same configuration as the hydrogen it replaced. Hence the 11α-hydrogen is transformed to the 11α-hydroxyl and the 11β-hydrogen into the 11β-hydroxyl group. The above findings were further confirmed by the use of deuterium labelled substrates. Corey et al.[26] incubated 11β-deuterated progesterone [28] with Rhizopus nigrans and obtained 11α-hydroxypregn-4-ene-3,20-dione-11β$^2$H [29] (label retained).

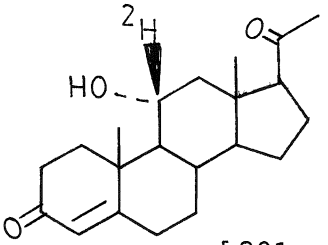
From the above studies it seems possible that hydroxylation at saturated carbons of the steroid nucleus proceeds by direct electrophilic insertion of an oxygen atom into a C-H bond as shown in Figure 5.

Figure 4. Use of tritiated substrates in elucidating the mechanism of 11α- and 11β-hydroxylation of steroids (see text for details).



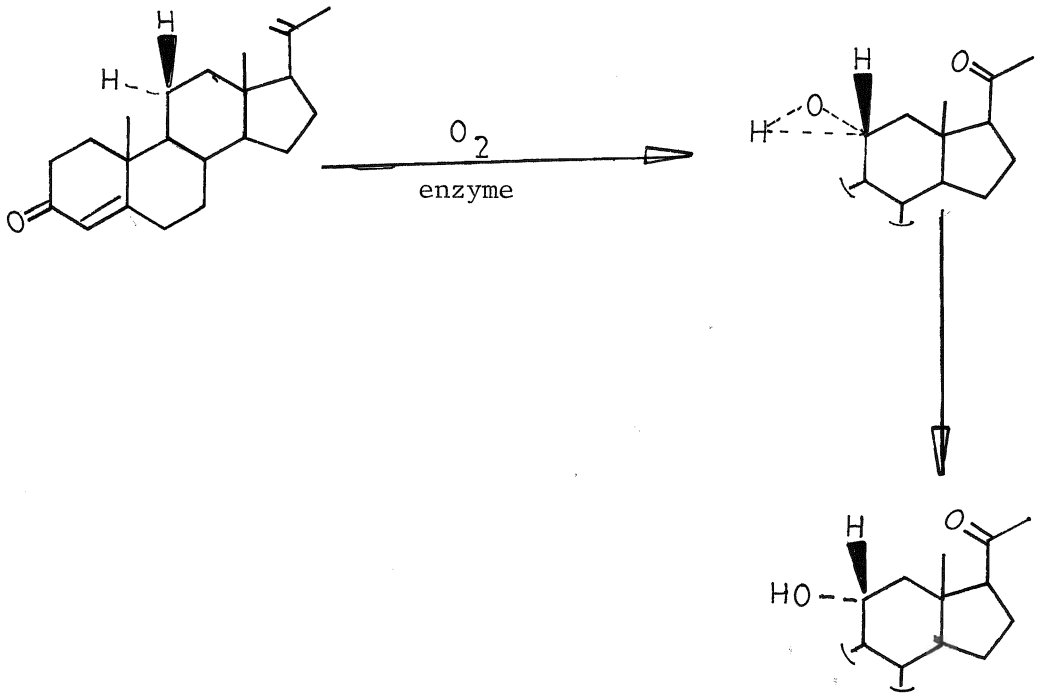



[28]




[29]

Figure 5. Stereospecific hydroxylation of steroids

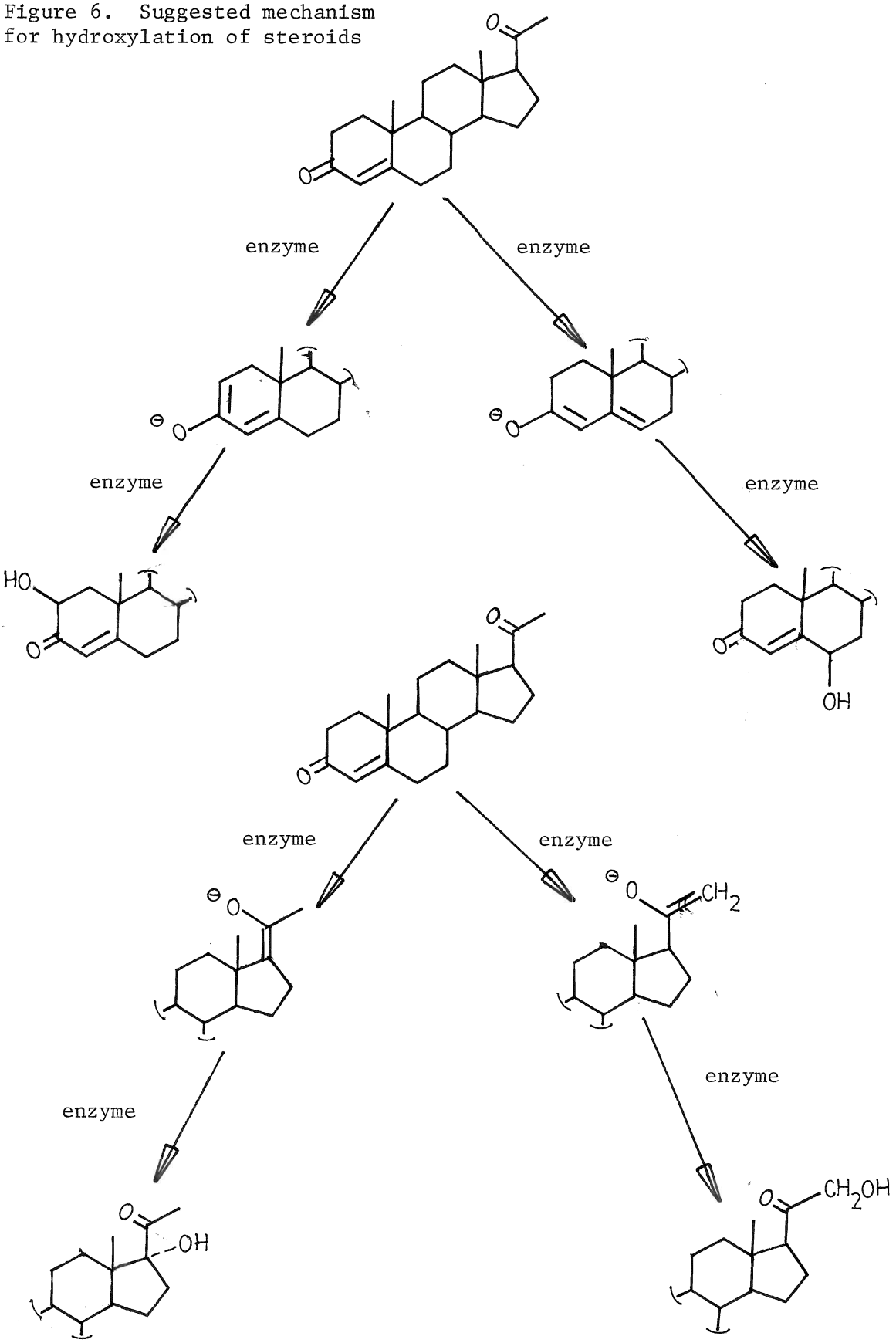


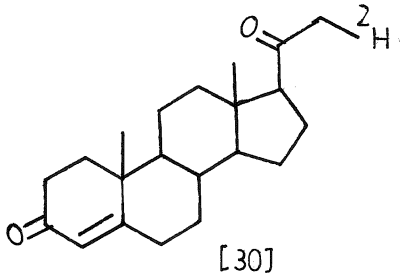
However, it has been suggested[18b] that at sites in the steroid nucleus adjacent or vinylogous to a carbonyl function, a quite different mechanism may be operative.

The proposal has been made that at the C-2,6,17 and 21-positions of $\Delta^4$3,20-diketo steroids, the carbon to be hydroxylated is first activated by enolization and then hydroxylation occurs by electrophilic attack of oxygen at the enol (Fig. 6). However, studies by Holland et al.[27] using the fungus Aspergillus niger, a C-21 hydroxylator, and 21-d-progesterone [30] as substrate did not support the above proposal of enzyme participation in enol formation during 21-hydroxylation. They found that either of the two prochiral hydrogens of [30] could be lost with equal facility; therefore, the products obtained from the incubation were a diastereomeric monodeutero-alcohol mixture and the unlabelled 21-hydroxyprogesterone [17]. Also, the magnitude of the deuterium isotope effect of 1.3 is not consistent with the proposal that an enolic intermediate is involved in C-21-hydroxylation.

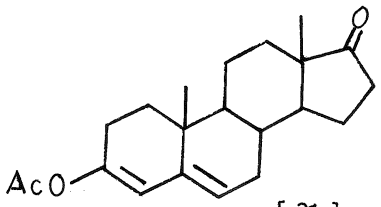
Further studies were carried out to investigate the above proposal using steroids having the $\Delta^4$-3-keto function and the fungus Rhizopus arrhizus, an organism known to hydroxylate the 6β-positions of $\Delta^4$-3-keto steroids.[28-30] The incubation of equimolar amounts of the enol acetate [31] and 16-d-androst-4-ene-3,17-dione (50% d) [32] with Rhizopus arrhizus provided 6β-hydroxyandrost-4-ene-3,17-dione [33] with a deuterium content of 18 percent. The observation was made that if [31] was hydrolysed to androst-4-ene-3,17-dione [10] prior to hydroxylation then a deuterium content of 25 percent or greater should be observed in

Figure 6. Suggested mechanism for hydroxylation of steroids

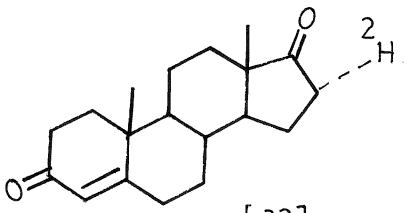




[30]



[31]



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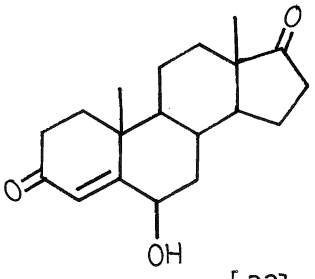


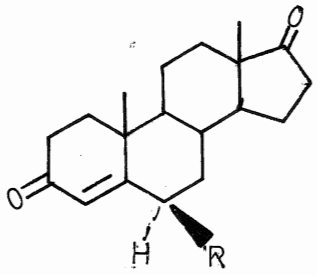
[33]

the product. The observed 18 percent strongly suggests that an enolic intermediate is involved since [31] is metabolized more rapidly than the parent substrate [10].[31]

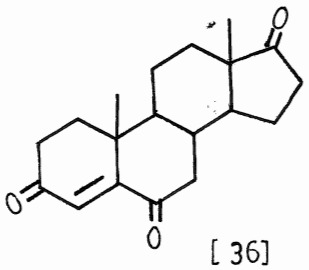
Further evidence supporting an enolic intermediate was obtained through the incubation of 6β- and 6α-halogenated substrates.[32] The incubation of 6β-chloroandrost-4-ene-3,17-dione [34] with R. arrhizus provided the 11α-hydroxylated product and small amounts of the 6α-chloride [38] along with some recovered starting material. In contrast, the incubation of the 6β-fluoro analogue [35] gave androst-4-ene-3,6,17-trione [36] and 3β-hydroxyandrost-4-ene-6,17-dione [37] among other products. The corresponding 6α-halo substrates were recovered largely unchanged although small amounts of [36] were obtained in each case. The products [36], [37] and [38] were rationalized as arising from protonation, electrophilic oxidation and/or hydration of the halo-enol intermediates as shown in Figure 7.

More evidence (be it circumstantial) supporting an enolic intermediate during 6β-hydroxylation of $\Delta^4$-3-keto steroids is gained from the observation that incubation of a large number of steroids having the $\Delta^4$-3-keto function with R. arrhizus invariably gave a 6β-hydroxylated steroid among the products[9,28,29,30] whereas incubation of 17β-hydroxyandrostan-3-one [39] (C4-C5 double bond absent) with the same fungus gave only the 11α- and 6α-monohydroxylated products.[34] Studies investigating whether hydroxylation at the C2 and C17 positions occurs through an enolic intermediate have not been encountered.

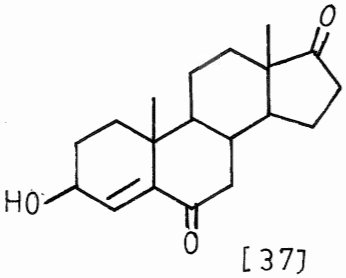
Until recently, the majority of the substrates investigated in fungal transformation of steroids possessed the $\Delta^4$-3-keto function. This was due



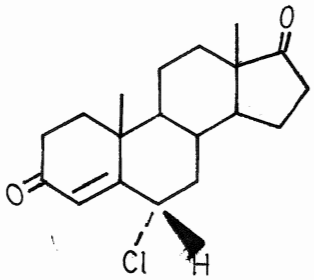
[34] R= Cl
[35] R= F



[36]

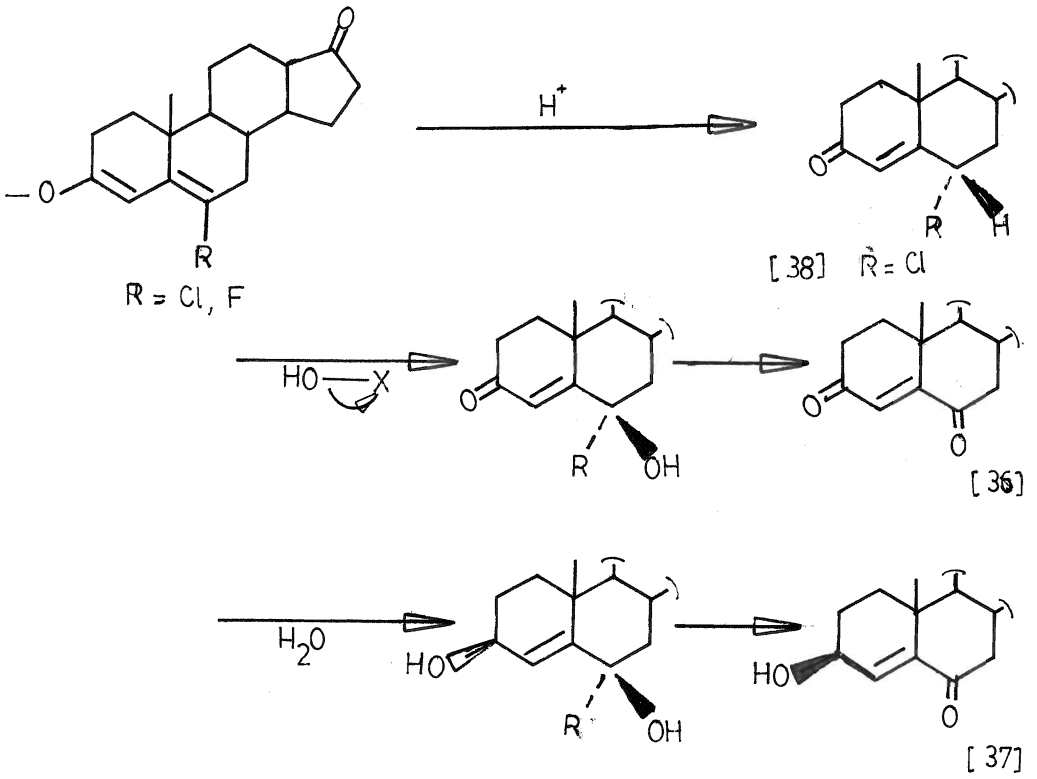


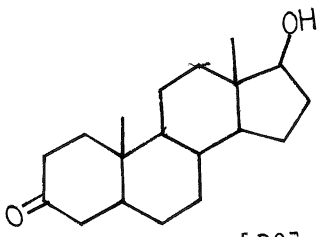
[37]



[38]

Figure 7. Proposed mechanism for the metabolism of 6-halo-androst-4-ene-3,20-dione by Rhizopus arrhizus



almost entirely to the medicinal importance of steroids having this function. The $\Delta^4$-3-keto function appears to have placed some restriction on the position of the steroid nucleus that could be hydroxylated. Thus many fungi have acquired labels indicative of the position they most frequently hydroxylate; the 11α-hydroxylator is an organism which frequently hydroxylates the 11α position.

Lately, however, a large number of steroidal products have been isolated from incubations with substrates lacking the $\Delta^4$-3-keto function.[35] From observation of these products, it became apparent that the position of hydroxylation in many cases may well depend on the position of the substituent in the steroid nucleus. It was observed that by systematically changing the positions of the oxygenated substituents around the steroid skeleton, it was sometimes possible to direct hydroxylation to certain positions.[36-38] The pattern of hydroxylation is therefore influenced by the substituents present in the substrate. This point is illustrated by the observation that incubation of di-oxygenated 5α-androstanes having a carbonyl at C3 with the fungus Calconectria decora gave products hydroxylated in ring D mainly at C15. On the other hand, substrates having the carbonyl at C17 provided products hydroxylated at carbon-1 and -6.[37]

Other fungi have a marked tendency to hydroxylate at a certain position irrespective of the substituents present. One such fungus is Aspergillus ochraceus which 11α-hydroxylates many different substrates and only hydroxylates other positions if carbon-11 is occupied.[39]

One suggestion[40,41] that has been put forward regarding the pattern of hydroxylation observed in many fungi is that the substrate may bind

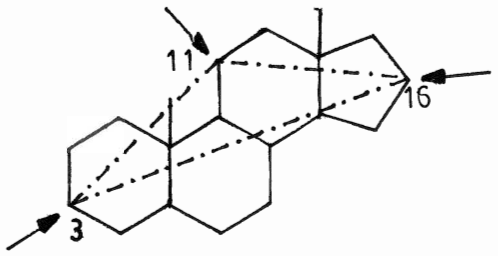
to the surface of the hydroxylating enzyme at any of three sites in a triangular arrangement outlined in Figure 8. Each site has both binding and hydroxylating capabilities. The substrate's keto or hydroxy function may bind to one or two sites (depending on how many oxy groups are present). The enzyme then proceeds to hydroxylate the C-H bond(s) that approach the vicinity of the third site.

This model, although simplistic, is supported by the observation that many fungi have a great propensity for hydroxylating apical positions of the triangle. There is also the tendency for many fungi to di-hydroxylate mono-oxygenated substrates and mono-hydroxylate di-oxygenated substrates at the apices of the triangle or at sites close to them.

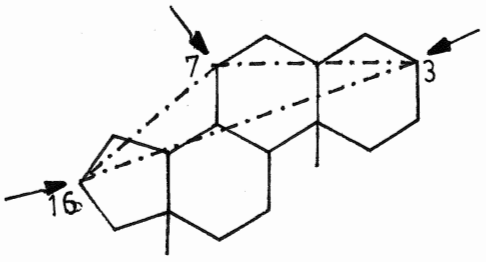
A fairly large accumulation of evidence suggests that many enzymes responsible for steroidal hydroxylations are inducible. It has been shown that certain substrates were 11β- and 14α-hydroxylated with cell free extracts of the fungus Curvularia lunata only if the enzyme(s) responsible were first induced in the intact cells.[42-44]

It is sometimes necessary for the "right" structural features to be present in the substrate before a particular hydroxylation will occur. For example, it is necessary for di-oxygenated androstane substrates to possess an oxygen at carbon-11 before 6β-hydroxylated products are obtained when incubated with Aspergillus ochraceus.[39] Also, the introduction of a second double bond can influence the proportion and types of products obtained: rac-17β-hydroxyestra-4,8(14)-diene-3-one [40] incubated with C. lunata afforded rac-7α,10β- and 11β-mono-hydroxylated products,[45] whereas rac-17β-hydroxyestra-4-ene-3-one [41] provided resolved 10β-, 12α- and 14α-monohydroxy and racemic 6β- and

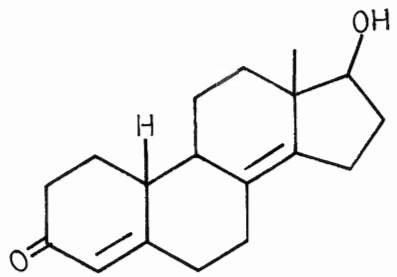
Figure 8. Arrows indicate approximate positions where the steroid may bind to or be hydroxylated by the enzyme. The steroid may bind in the normal (A) or reverse (B) phase.



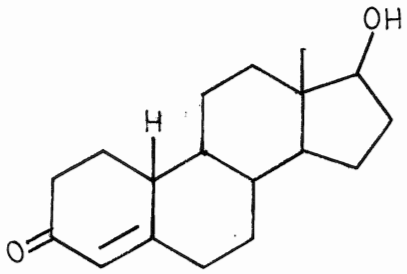

(A)




(B)




[40]




[41]

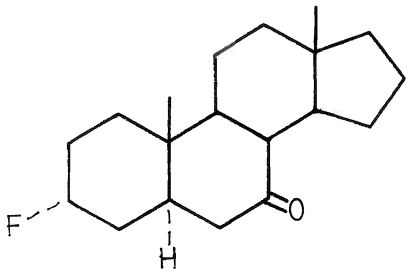
7β-monohydroxy derivatives.[46]

The majority of the studies dealing with substituent effects on the pattern of hydroxylation were carried out with oxygenated groups being the substituent of interest. Although many halogenated steroids have been used as substrates, only a few systematic studies have dealt with the effect halogenated substituents have on the pattern of hydroxylation[47,48] and there have been even fewer studies dealing with the effect halogen substitution has on the metabolism of medicinally important $\Delta^4$-3-keto steroids.

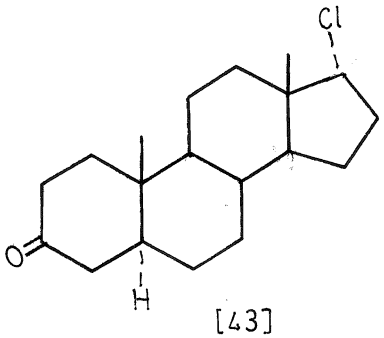
The intent of the present study was to investigate the effect placement of the halogen at favoured sites of hydroxylation had on the pattern of fungal metabolism of $\Delta^4$-3-keto steroids.

The first systematic study dealing with the effects halogeno substituents had on fungal metabolism of steroids was undertaken by Jones and coworkers.[47] They observed that the microbiological effect of the halogeno substituent depends on its nature, position and configuration in the steroid nucleus. The effect of configuration was illustrated by the observation that incubation of 3α-fluoroandrostan-7-one [42] with the fungus Calconectria decora provided products hydroxylated at the 1β- and 6β-positions similar to the parent ketone whilst the 3β-isomer was poorly metabolized.

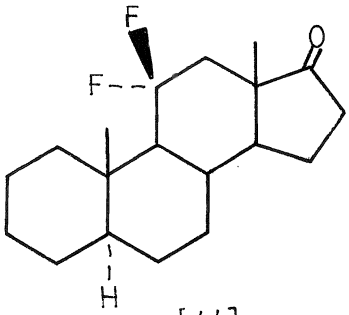
The metabolism of the 3α-chloro and 3α-bromo analogues resembles that of the 3α-fluoro derivative while the 3β-chloro and -bromo isomers were utilized more rapidly than the parent ketone. These observations exemplify the influence of the nature of the halogen on the metabolism of the substrate. It was also observed that 17α-chloro-5α-androstan-3-one [43]




[42]




[43]




[44]

is not metabolized by the same fungus, whereas the 17α-fluoro analogue is dihydroxylated at the 12β- and 15α-positions similar to the parent ketone.

In a subsequent study by the same group,[48] it was observed that introduction of a fluorine atom at favoured sites of hydroxylation resulted (with few exceptions) in hydroxylation at sites remote from the fluorine atom. This point is illustrated by the finding that incubation of 12,12-difluoro-5α-androstan-17-one [44] with Aspergillus ochraceus (a fungus with a marked propensity for 11α-hydroxylation) gave the 7α-hydroxylated product. Thus the conclusion was made that the tendency of the fluorine atom to inhibit hydroxylation at or adjacent to the carbon atom to which it is bonded outweighs the directing effects of the keto group or the site specificity of the micro-organism. It was also observed that a fluorine atom at a site remote from the favoured site of oxidation facilitates hydroxylation.

In the present study, $\Delta^4$-3-keto steroids, halogenated and in one case methylated at favoured sites of hydroxylation were prepared and incubated with three different fungi. As mentioned before, it was of interest to observe any effects the halogens might have on the pattern of metabolism of such substrates. Although many medicinally important $\Delta^4$-3-keto-halogenated steroids have been used successfully in excess of two decades, there is a scarcity of information regarding the metabolism of these steroids.

# EXPERIMENTAL

## Apparatus, Materials and Methods

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 237B grating infrared spectrophotometer, $^{1}H$ NMR spectra with a Bruker WP-60 or a Varian A60 using $CDCl_3$ or DMSO-$d_6$ as solvent and TMS as the internal standard. 15.08 MHz $^{13}C$ NMR spectra were recorded with a Bruker WP-60 using $CDCl_3$ or DMSO-$d_6$ as solvent and TMS as internal standard. Mass spectra were obtained with an AEI MS30 double beam mass spectrometer at 70 eV. Column chromatography was performed on silica gel, thin layer chromatography on Merck silica gel 60-F254 (0.25 mm thick). The plates were examined under UV light or sprayed with a 80% concentrated sulfuric acid/ethanol solution and developed at 110°C. Preparative tlc was performed on Merck silica gel F254 (2.0 mm thick) and was examined under UV light. Elemental analyses were performed by Guelph Chemical Laboratories Ltd., Guelph, Ontario.

Preparation of steroidal substrates

21-Methanesulfonyloxypregn-4-ene-3,20-dione [45][51]

5 g (0.015 moles) of 21-hydroxypregn-4-ene-3,20-dione [17] was added to 12 mL of dry pyridine and the mixture cooled to -10°C. 1.73 g (0.015 moles) of methanesulfonyl chloride was added and the mixture stirred below -5°C for 1.25 hours. The reaction was stopped by the addition of ether and water. The white precipitate was filtered and recrystallized from methanol giving 4.2 g (67% yield) of the title compound, m.p. 157-160°C (lit.[52] 156-157°C). The $^1$H NMR spectrum included signals at δ 0.75 (3H,s C18H), 1.23 (3H,s C19H), 3.27 (3H,s methyl of sulfonate group), 4.78 (2H,s C21H), 5.73 (1H,s C4H). Mass spec. M/e (%): 408 (5), 393 (2), 366 (10), 330 (20), 299 (100).

21-Chloropregn-ene-3,20-dione [46]

The procedure followed in the preparation of [45] was also used in the preparation of this substrate except in this case the reaction was performed at room temperature. 5.0 g (0.015 moles) of 21-hydroxypregn-4-ene-3,20-dione [17] was added to 10 mL of dry pyridine. The mixture was cooled to 0°C and 2.16 g (0.019 moles) of methanesulfonyl chloride was added. The mixture was stirred while warming to room temperature for two hours, at which time the product had precipitated and the solution had acquired a pinkish colour. Water was added and the chloride filtered. Recrystallization was effected from methanol (3 times) to give 3.8 g (73% yield) of the title compound, m.p. 203-205°C (lit.[53] 206-208°C). The $^1$H NMR spectrum included signals at δ 0.71 (3H,s C18H), 1.20 (3H,s C19H), 4.12 (2H,s C21H), 5.75 (1H,s C4H). Mass spec.

M/e (%): 350 (8), 348 (25), 333 (8), 314 (16), 312 (21), 299 (100). $^{13}C$ NMR data is located in Table 1.

21-Fluoropregn-4-ene-3,20-dione [47][54]

4 g (0.69 moles) of potassium fluoride and 6 g (0.023 moles) of 18-crown-6-ether (1,4,7,10,13,16-hexaoxacyclooctadecane [73], obtained from Aldrich Chemical Company) were suspended in 25 mL of freshly distilled DMF. 3.5 g (0.0085 moles) of 21-methanesulfonyloxypregn-4-ene-3,20-dione [45] was added and the mixture refluxed at water aspirator pressure for 21 hours. Water and ether were added and the precipitated product filtered. The product was chromatographed on a silica gel column by stepwise elution from benzene to ether (increments of 10% ether). The title compound was recrystallized from acetone giving 1.0 g (35% yield), m.p. 138-140°C (lit.[16] 141.5-142.2°C). The $^{1}H$ NMR spectrum included signals at $\delta$ 0.72 (3H,s C18H), 1.20 (3H,s C19H), 4.79 (2H,d C21H), 5.82 (1H,s C4H). Mass spec. M/e (%): 332 (100), 317 (24), 299 (26), 290 (100). $^{13}C$ NMR data may be found in Table 1.

21-Bromopregn-4-ene-3,20-dione [48][51]

2.5 (0.006 moles) of 21-methanesulfonyloxypregn-4-ene-3,20-dione [45] was added to 15 mL of acetone. After the solution was complete 3.5 g (0.63 moles) of lithium bromide was added and the mixture refluxed with stirring for 2 hours. After cooling, the white solid was collected, dissolved in chloroform, washed with water and dried (anhydrous sodium sulfate). After removal of the chloroform, recrystallization was

effected from acetone giving 1.8 g (76% yield) of the title compound, m.p. 159-162°C (lit.[56] 190-191°C). The $^{1}H$ NMR spectrum included signals at δ 0.72 (3H,s C18H), 1.20 (3H,s C19H), 3.96 (2H,s C21H), 5.79 (1H,s C4H). Mass Spec. M/e (%): 394 (16), 392 (17), 352 (28), 350 (27), 313 (53), 299 (100). $^{13}C$ NMR data are located in Table 1.

Pregn-4,9(11)-diene-3,20-dione [49][57]

15 g (0.045 moles) of 11α-hydroxypregn-4-ene-3,20-dione [18] was dissolved in 85 mL of methylene chloride and 14.5 g (0.076 moles) of diethyl-(2-chloro-1,1,2-trifluoroethyl)amine [50] was added. After 24 hours at -20°C, the solvent was removed under reduced pressure and recrystallization of the product effected from 95% ethanol giving 10 g (first crop). A second crop provided 1.6 g giving a total yield of 11.6 g (81%), m.p. 122-124°C (lit.[57] 123-125°C). The $^{1}H$ NMR spectrum included signals at δ 0.65 (3H,s C18H), 1.35 (3H,s C19H), 2.14 (3H,s C21H), 5.55 (1H,t C11H), 5.73 (1H,d C4H). Mass spec M/e (%): 312 (89), 297 (100), 269 (28). $^{13}C$ NMR data is located in Table 1. Note: tlc shows this reaction is complete after 15 minutes when performed at room temperature.

9α-Bromo-11β-fluoropregn-4-ene-3,20-dione [51][59]

To 40 mL of dry tetrahydrofuran was added 50 mL of the 70% HF/pyridine reagent. The mixture was cooled to -50°C and 3.5 g of N-bromosuccinimide followed by 5.5 g of [49] in 10 mL of THF was added. The mixture was stirred at the same temperature for one hour and then

allowed to sit at room temperature for another hour, after which cold saturated sodium carbonate solution was added cautiously until the solution was slightly basic. The product was extracted with methylene chloride, and after drying (sodium sulfate) and removal of the methylene chloride, the product was recrystallized from methanol giving 6.1 g (84% yield), m.p. 143-145°C (lit.[60] 145-150°C). The $^{1}H$ NMR spectrum included signals at δ 0.81 (3H,d C18H), 1.39 (3H,d C19H), 2.14 (3H,s C21H), 5.15 (1H,m 11αH), 5.69 (1H,s C4H). Mass spec. M/e (%): 412 (7), 410 (8), 331 (19), 312 (100), 269 (24). $^{13}C$ NMR data are presented in Table 1.

11β-fluoropregn-4-ene-3,20-dione [52][61]

To 25 mL of dry THF was added 2.8 g of [51], 10 mL of tri-n-butyltin hydride and 10 mg of azoisobutyronitrile. The mixture was allowed to sit at room temperature for five hours, after which time a few drops of water were added and the solvent removed under reduced pressure.

Recrystallization of the residue from an 85% ethanol/water mixture provided 1.95 g (86% yield) of the title compound, m.p. 169-172°C (lit.[62] 156-158°C). The $^{1}H$ NMR spectrum included signals at δ 0.81 (3H,d C18H), 1.36 (3H,d C19H), 2.14 (3H,s C21H), 5.13 (1H,m 11αH), 5.71 (1H,s C4H). Mass spec. M/e (%): 332 (54), 317 (10), 312 (100), 304 (16), 297 (46). The $^{13}C$ NMR data is presented in Table 1.

Androst-4-ene-3-one-17β-carboxylic acid [53][63]

2.0 g of 21-hydroxypregn-4-ene-3,20-dione [17] was added to 25 mL of acetone containing one drop of sulfuric acid. 2.0 g of paraperiodic acid ($H_5IO_6$) in 6 mL of water was then added and the solution refluxed for 12 hours. The acetone was removed under reduced pressure and chloroform (50 mL) added. The chloroform solution was then extracted with 4% sodium hydroxide solution (3 times). Fresh chloroform was added to the aqueous extract and the mixture acidified with dilute hydrochloric acid. The mixture was then extracted with chloroform (3 times) and after drying (sodium sulfate), the chloroform was removed to give 1.4 g (73% yield) of the title compound, m.p. 246-250°C (lit.[64] 246-250°C). The $^1H$ NMR spectrum included signals at δ 0.75 (3H,s C18H), 1.20 (3H,s C19), 5.65 (1H,s C4H). Mass spec. M/e (%): 316 (57), 301 (18), 298 (13), 283 (14), 274 (100). $^{13}C$ NMR data are presented in Table 1.

21-Methylpregn-4-ene-3,20-dione [55][65,66]

To 30 mL of dry benzene was added 0.6 g of the previously prepared acid [53]. Nitrogen was bubbled through the suspension for 15 minutes and after cooling the mixture to 5°C, 2mL of oxalyl chloride was added. The mixture was stirred while allowing to warm to room temperature and after 45 minutes, the solution was complete. The solvent was removed under vacuum and the residual acid chloride [54] was dried *in vacuo* for a further 6 hours. Dry hexamethylphosphoric triamide (HMPA) (10 mL) was added to the residue and after the solution was complete, 0.56 g of tetraethyltin and 10 mg of benzylchlorobis(triphenylphosphine) palladium II [56] was added. The mixture was placed in a water bath at 80°C and

heating continued at this temperature for 45 minutes. The darkened mixture was cooled to room temperature and water added. The products were extracted with ether and after washing the ether solution with 2% sodium hydroxide and drying with anhydrous sodium sulfate, the solution was concentrated. The residue was chromatographed on preparative tlc silica gel plates using 30% ethyl acetate/benzene solution as the developing solvents. The desired product was recrystallized from methanol/water solution to give 0.21 g (42% yield) of the title compound, m.p. 139-141°C (lit.[67] 151-152°C). The $^{1}H$ NMR spectrum included signals at $\delta$ 0.66 (3H,s C18H), 1.19 (3H,s C19), 1.04 (3H,t C22H), 5.74 (1H,s C4H). Mass spec. M/e (%): 328 (64), 313 (24), 299 (59), 283 (73), 271 (100). $^{13}C$ NMR data are presented in Table 1.

3,20-Bisethylene ketal of Pregn-4-ene-3,11,20-trione [56][68]

To 100 mL of toluene was added 10.1 g of pregn-4-ene-3,11,20-trione [57], 15 mL of ethylene glycol and 0.17 g of p-toluenesulfonic acid monohydrate. The mixture was refluxed with stirring while the water formed during the reaction was removed by a Dean Stark water trap. After 20 hours, the mixture was cooled and 50 mL of methanol containing 1 g of KOH was added. The organic layer was washed with water (3 times) and the washings further extracted with chloroform. The combined mother liquor and the chloroform extract were dried (anhydrous sodium sulfate) and after removal of the solvent, recrystallization was effected from methanol giving 9.1 g of the title compound. M.p. 177-179°C (lit.[68] 176-179°C). The $^{1}H$ NMR spectrum included signals at $\delta$ 0.73 (3H,s C18H),

1.22 (3H,s C19H), 1.25 (3H,s C21H), 3.93 (8H,s ethylene H), 5.30 (1H,s C6H). Mass spec. M/e (%): 416 (100), 401 (34), 388 (17), 372 (21), 357 (24), 328 (13).

11β-Hydroxypregn-4-ene-3,20-dione [58][58]

8.9 g of the above prepared bisethylene ketal [56] was added to 400 mL of a 80% aqueous t-butanol solution. The mixture was warmed until the solution was complete and then cooled to room temperature. 9.36 g of sodium borohydride was added and stirring continued at room temperature for 24 hours. The solvent was then removed under reduced pressure and chloroform (200 mL) was added to the residue. After washing (water) and drying (anhydrous sodium sulfate), the chloroform was removed. To the residue was added 250 mL of methanol and 0.5 mL of concentrated sulfuric acid. The mixture was refluxed for 3 hours and then cooled to room temperature and the solution neutralized by the addition of saturated sodium hydrogen carbonate solution. The product was extracted (3 times) with chloroform and after drying (anhydrous sodium sulfate) the solvent was removed. The product was recrystallized from a methanol/water mixture to give 6.5 g of the title compound, m.p. 185-188°C (lit.[68] 186-188°C). The $^{1}H$ NMR spectrum included signals at δ 0.91 (3H,s C18H), 1.45 (3H,s C19H), 2.12 (3H,s C21H), 4.39 (1H,m 11αH), 5.66 (1H,s C4H). Mass spec. M/e (%): 330 (16), 312 (22), 297 (17), 269 (10), 124 (100).

3-Ethoxyandrosta-3,5-diene-17-one [59][69]

To 15 mL of dry dioxane was added 2.0 g of androst-4-ene-3,17-dione, 2 mL of triethyl orthoformate and 0.15 g of p-toluenesulfonic acid monohydrate. The mixture was stirred at room temperature for 1.5 hours after which time the solution had darkened. One mL of pyridine and 10 mL of water were added and the mixture cooled in an ice/water bath for several hours. After precipitation was complete, the solid was filtered and the product recrystallized from methanol containing a trace of pyridine to give 1.82 g of the title compound, m.p. 150-152°C (lit.[70] 152°C). The $^{1}H$ NMR spectrum included signals at $\delta$ 0.91 (3H,x C18H), 1.00 (3H,s C19H), 1.30 (3H,t methyl of ethoxy group), 3.78 (2H,q methylene of ethoxy group), 5.17 (2H,m C4H, C6H).

Androst-4,6-diene-3,17-dione [60][69]

4.65 g of 3-ethoxyandrosta-3,5-diene-17-one [59] was added to 170 mL of 95% aqueous acetone solution. When the solution was complete, 3.6 g of 2,3-dichloro-5,6-dicyanoquinone (DDQ) [61] in 30 mL of 95% aqueous acetone was added portionwise. After the addition of DDQ was complete (3 min), the reaction mixture was allowed to stir for a further 5 minutes. After the addition of 70 mL of acetone, the solvent was removed and 200 mL of chloroform added. The chloroform solution was cooled and then filtered. The filtrate was washed with water (3 times), 1% aqueous sodium hydroxide solution (3 times) and water (3 times). The chloroform solution was dried (sodium sulfate) and concentrated. The crude product was filtered through an alumina column (neutral, 100 g)

eluted with acetone. The product was recrystallized from ethyl acetate giving 3.0 g of the title compound, m.p. 169-171°C (lit.[69] 169-170°C). The $^{1}H$ NMR spectrum included signals at δ 0.97 (3H,s C18H), 1.13 (3H,s C19H), 5.72, 6.12 (2H,s C6H, C7H), 6.12 (1H,s C4H). Mass spec. M/e (%): 284 (100), 269 (17), 254 (9). $^{13}C$ NMR data are presented in Table 1.

6α,7α-Epoxyandrost-4-ene-3,17-dione [62]

The procedure described by Hossain et al.[71] was used in this preparation and the one to follow: 2.35 g of androst-4,6-diene-3,17-dione [60] was added to 30 mL of dry dichloromethane. 2.20 g of m-chloroperbenzoic acid was then added portionwise over a ten minute period with stirring. After the solution was complete, 0.6 g of anhydrous sodium sulfate was added and stirring continued at room temperature for 21 hours. The precipitated m-chlorobenzoic acid was filtered and the filtrate cooled to -20°C. After a second filtration, the filtrate was diluted with more dichloromethane and washed 3 times with 10% sodium hydrogen carbonate solution and then 3 times with water. The dichloromethane solution was dried (anhydrous sodium sulfate) and concentrated. The residue was filtered through a neutral alumina column (120 g) eluted with ethyl acetate. The crude mixture was then chromatographed on a silica gel column (90 g), eluted from benzene to ether (10% increments of ether). The components obtained from the column included androst-4,6-diene-3,17-dione [60] (0.6 g) identical to starting material, 6α,7α-epoxyandrost-4-ene-3,17-dione [62] (0.23 g), m.p. 212-214°C (lit.[71] 216-222°C). The $^{1}H$ NMR spectrum included signals

at δ 0.95 (3H,s C18H), 1.13 (3H,s C19H), 3.51 (2H,s 6βH,7βH), 6.14 (1H,s C4H). Mass spec. M/e (%): 300 (100), 287 (16), 284 (42), 269 (26). $^{13}C$ NMR data are presented in Table 1. Other unidentified polar products were also recovered.

7α-Hydroxyandrost-4-ene-3,17-dione [63][71]

0.30 g of 6α,7α-epoxyandrost-4-ene-3,17-dione was dissolved in 10 mL of 95% ethanol. 0.3 mL of 10% sodium hydrogen carbonate solution was then added and the mixture cooled to -5°C (ice/sodium chloride). 3.1 g of freshly prepared amalgamated aluminum was added. (The aluminum amalgam was prepared in the following manner: alumina 5g (small pellet from Matheson, Coleman and Bell) was added to a solution of mercuric chloride (1.7 g in 20 mL of water) containing hydrochloric acid (0.5 mL). The mixture was shaken for 5 minutes and the liquid decanted. The amalgam was washed with distilled water until the washing was neutral and used immediately.) After stirring below 0°C for 6 hours, tlc showed the reaction was complete. Chloroform (10 mL) was added and the mixture filtered. The filtrate was concentrated and residue chromatographed on silica gel (25 g) eluted from benzene to ethyl acetate (10% increments of ethyl acetate). After recrystallization from acetone, 0.21 g of the title compound was obtained, m.p. 227-230°C (lit. 220-222.5°C[72], 240-245°C[71]). The $^{1}H$ NMR spectrum included signals at δ 0.92 (3H,s C18H), 1.22 (3H,s C19H), 4.10 (1H,bs 7β-H), 5.79 (1H,s C4H). Mass spec. M/e (%): 302 (15), 284 (100), 269 (29). $^{13}C$ NMR data are presented in Table 1.

The following procedures are a few of the many tried in the attempted preparation of 7-halo-androst-4-ene-3,17-dione:

Attempted preparation of androst-4-ene-3,17-dione-7α-p-toluene sulfonate[84]

20 mg of 7α-hydroxyandrost-4-ene-3,17-dione [63] was added to 2 mL of dry pyridine. After cooling this mixture to 0°C, 0.1g of freshly purified p-toluenesulfonyl chloride was added. The reaction mixture was then allowed to sit at room temperature for 72 hours at which time tlc showed the presence of two components. The pyridine was removed under reduced pressure and chloroform added. After washing with water, 5% aqueous hydrochloric acid and 2% aqueous sodium hydroxide, the chloroform solution was dried (anhydrous sodium sulfate) and concentrated. Analysis of the residue by $^1$H NMR and mass spec. showed it to be a mixture of the starting material and androst-4,6-diene-3,17-dione [60]. When the above reaction was performed below -5°C, no reaction occurred.

Attempted preparation of 7α-chloroandrost-4-ene-3,17-dione[85]

12 mg of 7α-hydroxyandrost-4-ene-3,17-dione was added to 2 mL of dry chloroform. Thionyl chloride (10 drops in 1 mL of chloroform) was added with stirring at room temperature. After 30 minutes, tlc showed two components present, neither of which was the starting material. After the addition of chloroform (20 mL), the mixture was washed (water), dried (anhydrous sodium sulfate) and the chloroform evaporated. $^1$H NMR and mass spectral analysis of the residue showed that only the 6-dehydro elimination product [60] was present. When thionyl chloride was used as

the solvent in the above, reaction, carried out at 0°C, a complex mixture of products was obtained, none of which was the expected 7α-chloride.

Attempted preparation of 7α-methanesulfonyloxyandrost-4-ene-3,17-dione[86]

0.1 g of 7α-hydroxyandrost-4-ene-3,17-dione was added to 5 mL of pyridine. 0.18 g of methanesulfonyl chloride was added and the mixture stirred at room temperature for 5 hours. Ether was added and the mixture washed with water (4 times), then dried (anhydrous sodium sulfate). The ether was rotary evaporated and the crude residue subjected to $^1$H NMR and mass spectral analysis which showed only the 6-dehydro-steroid [60] was present. When the above reaction was attempted at -20°C for 72 hours, no reaction occurred.

Attempted preparation of 7α-chloroandrost-4-ene-3,17-dione[87]

33 mg of sodium chloride was added to 4 mL of the 70% HF/pyridine reagent in a polyethylene bottle. 15 mg of 7β-hydroxyandrost-4-ene-3,17-dione was added to this mixture with stirring at 0°C. The reaction mixture was stirred at room temperature for 29 hours, after which time the dark mixture was poured into ice water and then neutralized with saturated sodium carbonate solution. The mixture was extracted with methylene chloride and after drying (anhydrous sodium sulfate), the methylene chloride was evaporated. Tlc showed two components were present. $^1$H NMR and mass spectral analysis further confirmed that both starting materials and the 6-dehydro steroid [60] were present, [60] being the major component.

Attempted preparation of 7α-bromoandrost-4-ene-3,17-dione[88]

0.1 g of androst-4,6-diene-3,17-dione in 2 mL of glacial acetic acid was cooled to 16°C. 1 mL of 48% HBr was added and the mixture stirred in an icewater bath for 0.5 hours. At this time, tlc showed 2 components present, however, when methylene chloride was added and the mixture was allowed to warm to room temperature, only the starting material was observed when analysed by tlc and $^1$H NMR spectroscopy. The same results as those above were obtained when anhydrous hydrochloric acid replaced hydrobromic acid and THF was used as the solvent.

Attempted preparation of 7α-chloroandrost-4-ene-3,17-dione[89,90]

To a stirred solution of 44 mg of N-chlorosuccinimide in 2 mL of dry THF, was added 73 mg of triphenylphosphine in 2 mL of dry THF. To this suspension was added 42 mg of 7β-hydroxyandrost-4-ene-3,17-dione. After stirring at room temperature for 1 hour, the mixture became clear and tlc indicated the presence of two components. The solvent was then removed under reduced pressure and chloroform added. The chloroform was washed (water), dried (anhydrous sodium sulfate) and evaporated. $^1$H NMR and mass spectral analysis of the crude residue indicated the presence of at least two steroidal products, none of which was either the starting material or the expected 7α-chloro steroid. The products were not further identified.

Preparation of reagents

2-Chloro-1,1,2-trifluorotriethylamine [50][57,73]

The procedure used was that described by Knox et al.[57] 2-chloro-1,1,2-trifluoroethylene was bubbled into 40 mL of diethylamine in a large test tube maintained between -5°C and -10°C for 8 hours. The crude product was distilled in vacuo to give 35 g of a colourless liquid (48% yield). It was necessary to distill off the coloured impurities before the product was collected. The $^1H$ spectrum showed signals at δ 1.20 (6H,t $CH_3$), 2.95 (4H,q $CH_2$) and 6.27 (1H,d CHClF).

2,3-Dicyanohydroquinone [74][79]

Concentrated sulfuric acid (50 mL) in 95% ethanol (200 mL) was added to a stirred suspension of benzoquinone (40 g) in 95% ethanol (900 mL). The temperature was kept between 24 and 28°C during the addition of potassium cyanide (80 g) in water (200 mL) over 45 minutes. The potassium cyanide solution was added until the light brown mixture turned dark brown and alkaline to litmus paper. The mixture was just acidified and the potassium sulfate filtered and washed with hot 95% ethanol (200 mL). The combined filtrate was concentrated under reduced pressure to about 200 mL without excessive heating.

The residue was cooled on ice and the crystals (black) filtered. Recrystallization from water with charcoal provided 21 g (68%) of 2,3-dicyanohydroquinone, m.p. 260°C. The $^1H$ NMR spectrum showed signals at δ 2.31 (2H,bs OH), 7.02 (2H,s ArH). Mass spec. M/e (%): 160 (100), 132 (27).

2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) [61][80]

10 g (0.062 moles) of [74] was added to a solution of water (70 mL) and concentrated hydrochloric acid (70 mL). The resultant suspension was treated with 19 g (0.15 moles) of nitric acid (70%) over 45 minutes with stirring at 35 ± 3°C. After the addition of the nitric acid, the yellow suspension was stirred for another hour, then filtered and washed with carbon tetrachloride. After drying *in vacuo* for 12 hours, 12.0 g of a bright yellow powder was obtained, m.p. 212-214°C (lit. 212-213°C,[82] 213-214°C[81]). Mass spec. M/e (%): 230 (57), 228 (100), 226 (57), 202 (19), 200 (37), 198 (16), 172 (31), 170 (39).

Benzylchlorobistriphenylphosphinepalladium(II) [56][82]

0.2 g of tetrakis(triphenylphosphine)palladium(0) [75] was added to a $N_2$-flushed 100 mL round-bottomed flask. 15 mL of dry benzene was flushed with nitrogen, then added to the flask. To the yellow suspension was added 0.42 g of distilled benzyl chloride in 1 mL of dry benzene also flushed with nitrogen. The mixture was stirred at room temperature for two hours and then the solvent was removed *in vacuo*. 30 mL of anhydrous ether was added to the residue and after 2 hours, the pale yellow crystals were filtered, washed 3 times with 15 mL portions of hexane to give 0.12 g of the title compound, m.p. 135-137°C (lit. 140-144°C,[82] 147-151°C[83]). The $^1$H NMR spectrum showed signals at δ 2.81 (2H,s benzylic H) and 6.71 (38H,m ArH).

Incubations with Aspergillus niger ATCC 9142[27]

Incubations were performed in one liter Erlenmeyer flasks, or a 5 L fermentation vessel, at 28°C. The one liter flasks were shaken at 180-190 rpm on a New Brunswick rotary shaker while the 5 L fermentation vessel was mechanically stirred and aerated with air sterilized by passage through a Whatman Gamma 10 filter.

The growth medium contained Czapex Dox nutrient (35 g/L) and was autoclaved at 15 pounds pressure and 120°C for 20 minutes. After cooling, the flasks were inoculated with spore suspensions obtained by shaking agar slopes of A. niger with sterile water. The flasks were incubated under the conditions mentioned above for 2 days, after which the various substrates listed below were added in 95% ethanol (20 mg/mL). After a further growth period, the mycelia were separated from the medium by filtration and both extracted with dichloromethane. The extracts were dried over anhydrous sodium sulfate and concentrated.

Pregn-4-ene-3,20-dione [11]

Incubation of 0.8 g of this substrate for control purposes for 4 days provided 0.51 g of crude extract which was chromatographed on a silica gel column (25 g) by stepwise elution from benzene to ether (increments of 10% ether) to give pregn-4-ene-3,20-dione [11] (0.20 g); spectral data identical with the starting substrate, and 21-hydroxypregn-4-ene-3,20-dione [17] (0.21 g) identified by spectral comparison with authentic material.

21-Fluoropregn-4-ene-3,20-dione [47]

Incubation of 0.8 g of this substrate for 5 days provided 0.71 g of crude extract. The crude extract was chromatographed on silica gel by stepwise elution from benzene to ether (10% increments of ether). 0.35 g of the starting material was recovered in addition to a small amount of an unidentified metabolite obtained in an impure form. The mass spectrum of this metabolite showed a molecular ion at m/e = 348 corresponding to the addition of one oxygen atom to the substrate. Further characterization was not attempted due to the limited quantity and the impure state of the material.

21-Chloropregn-4-ene-3,20-dione [46]

Incubation of 1.0 g of this substrate for 4 days gave 0.82 g of crude extract. Thin layer chromatography showed the main component to be substrate. The crude extract was chromatographed on 40 g of silica gel by stepwise elution from benzene to ether (10% increments of ether). The substrate (0.61 g) was the only component isolated from the column.

21-Bromopregn-4-ene-3,20-dione [48]

Incubation of 1.0 g of this substrate for 4 days provided 0.79 g of crude extract. Tlc showed the major component to be the substrate. The crude extract was further analysed by $^{1}H$ NMR which showed that the substrate was the only steroidal material present. Recrystallization of the crude extract from acetone provided pure 21-bromopregn-4-ene-3,20-dione.

21-Methylpregn-4-ene-3,20-dione [55]

Incubation of 0.16 g of this substrate for 4 days provided 0.12 g of crude extract. Tlc showed the major component to be the starting substrate. Further analysis by $^{1}H$ NMR showed only the starting material was present.

Incubation with Curvularia lunata NRRL 2380[46]

Vegetative cell cultures of C. lunata were grown in one litre erlenmeyer flasks consisting of the following components:

| | percent | per litre of water |
|---|---|---|
| Difco tryptone | 1.0 | 10 g |
| sucrose | 1.0 | 10 g |
| sodium nitrate | 0.2 | 2 g |
| dipotassium hydrogen phosphate | 0.1 | 1 g |
| magnesium sulfate heptahydrate | 0.05 | 0.5 g |
| potassium chloride | 0.05 | 0.5 g |
| ferrous sulfate heptahydrate | 0.001 | 0.01 g |

0.25 percent of calcium carbonate was added prior to sterilization at 15 pounds pressure, 120°C for 20 minutes.

Inoculations were made from 4% agar slopes of the fungus and the one litre flasks containing 180 mL of the medium was incubated at room temperature on a New Brunswick rotary shaker (180 rpm) for 2 days. The steroidal substrates listed below were then added in 95% ethanol and the fermentation continued for various time periods. The mycelia were then separated from the medium by filtration and both extracted with dichloromethane. The dichloromethane was then dried (anhydrous sodium sulfate) and concentrated. The crude residues were subjected to chromatography as necessary.

Pregn-4-ene-3,20-dione [1]

Incubation of 0.6 g of this substrate for control purposes for 24 hours provided 0.50 g of crude extract. After chromatographing the crude extract on silica gel by stepwise elution from benzene to ether (increments of 10% ether), the following components were identified: pregn-4-ene-3,20-dione [71] (0.15 g) identical with starting material, 11β-hydroxypregn-4-ene-3,20-dione [58] (0.12 g) identical in all respects to the chemically prepared material, 14α-hydroxypregn-4-ene-3,20-dione [64] (0.10 g), m.p. 184-188°C (lit.[18c] 192.5-194°C). The $^1$H NMR spectrum included signals at δ 0.79 (3H,s C18H), 1.21 (3H,s C19H), 2.12 (3H,s C21H), 5.68 (1H,s C4H). Mass spec. M/e (%): 330 (61), 312 (100), 297 (24), 269 (29), 149 (98). Also isolated was 11β,14α-dihydroxypregn-4-ene-3,20-dione [65] 0.05 g, m.p. 220-221°C (lit.[18d] 224-227°C). The $^1$H NMR spectrum included signals at δ 1.01 (3H,s C18H), 1.46 (3H,s C19), 2.12 (3H,s C21H), 4.35 (1H,m 11αH), 5.64 (1H,s C4H). Mass spec. M/e (%): 346 (1), 328 (1), 310 (1), 204 (4), 154 (100).

11β-fluoropregn-4-ene-3,20-dione [52]

Incubation of 0.7 g of this substrate for 60 hours gave 0.54 g of a crude extract. The crude extract was chromatographed on preparative tlc plates developed with 4% methanol in ether. The following components were identified: 11β-fluoropregn-4-ene-3,20-dione [61] (0.25 g), identical to starting substrate, 11β-fluoro-14α-hydroxypregn-4-ene-3,20-dione [66] (0.12 g), m.p. 193-195°C. The $^1$H NMR spectrum included signals at δ 0.89 (3H,d C18H), 1.37 (3H,d C19H), 5.11 (1H,m 11αH),

5.69 (1H,s C4H). Mass spec. $M^+$ found 348.210; calculated for $C_{21}H_{29}O_3F$ 348.210; M/e (%): 348 (6), 330 (36), 310 (36), 287 (43), 123 (100). $R_f$ values: 0.77 (benzene:ethyl acetate:acetone--3:3.5:3.5), 0.81 (benzene:acetone--3:7), 0.51 (acetone:hexane--8:2). Also identified was 11β-fluoro-14α,21-dihydroxypregn-4-ene-3,20-dione [67] (0.05 g) m.p. 175-178°C. The $^1H$ NMR spectrum included signals at δ 0.92 (3H,d C18H), 1.37 (3H,d C19H), 4.20 (2H,d C21H), 5.08 (1H,m 11αH), 5.70 1H,d C4H). Mass spec. $M^+$ found 364.206; calculated for $C_{21}H_{29}O_4F$ 364.205; M/e (%): 364 (1), 346 (2), 333 (27), 315 (100), 295 (9). $R_f$ values: 0.70 (benzene:acetone--3:7), 0.59 (benzene:ethylacetate:acetone 3:3.5:3.5), 0.29 (acetone:hexane--8:2).

Incubations with Rhizopus arrhizus ATCC 11145[76]

Rhizopus stolonifer ATCC 6227b[75]

These fungi were grown under the same conditions. The medium consisted of glucose (50 g), peptone (20 g) and corn steep liquor (3 ml) (Grand Island Biochemical Co.), per litre of distilled water. Incubations were performed in one litre Erlenmeyer flasks each containing 150 mL of the medium. The flasks were sterilized at 15 pounds pressure, 120°C for 20 minutes. After cooling, the flasks were inoculated with spore suspensions obtained by shaking agar slopes of the fungus with sterile water. The flasks were then incubated on a New Brunswick rotary shaker at room temperature for 60 hours after which time the mycelia were separated from the medium by filtration. The mycelia were washed with distilled water, macerated in a Waring blender with distilled water and aliquots re-suspended in distilled water (150 mL).

The steroidal substrates were then added in 95% ethanol (30 mg/ml) and incubation continued for various time periods under the same conditions outlined above. The mycelium and the water were then separated and both extracted with dichloromethane. The solvent was dried with anhydrous sodium sulfate and removed under reduced pressure. The crude residue was then subjected to chromatography when required.

11β-Fluoropregn-4-ene-3,20-dione

Incubation of 0.92 g of this substrate with R. stolonifer for 36 hours provided 1.61 g of crude extract. Tlc showed the medial fraction contained mainly one component with a lower $R_f$ value than the starting

material, while the mycelial fraction contained the unmetabolized substrate. Recrystallization of the medial extract provided 0.30 g of pregn-4-ene-3,11,20-trione [57], identical with both commercial and laboratory prepared material. Recrystallization of the mycelial extract gave 0.5 g of the starting substrate.

11β-Hydroxypregn-4-ene-3,20-dione

Incubation of 0.35 g of this substrate with R. stolonifer for 35 hours provided 0.4 g of crude extract. Tlc showed only one component. Recrystallization of the crude extract from methanol gave 0.30 g of pure pregn-4-ene-3,11,20-trione [57] also identical with authentic sample.

Androst-4-ene-3,17-dione

This incubation was performed to obtain 7β-hydroxandrost-4-ene-3,17-dione [70]. Incubation of 4.2 g of this substrate with R. stolonifer for 4 days provided 5.0 g of crude extract. The extract was chromatographed on silica gel by eluting with 70% ethyl acetate in benzene. The following components were identified: androst-4-ene-3,17-dione [10] (0.92 g), identical to the starting material, 6β-hydroxyandrost-4-ene-3,17-dione [33] (1.51 g), m.p. 189-191°C (lit.[29] 191-194°C). The $^1$H NMR spectrum included signals at δ 0.95 (3H,s C18H), 1.41 (3H,s C19H), 4.39 (1H,bs 6αH), 5.81 (1H,s C4H). Also identified was 11α-hydroxyandrost-4-ene-3,17-dione [69] (0.21 g), m.p. 220-223°C (lit.[29] 225-227°C). The $^1$H NMR spectrum included signals at δ 0.96 (3H,s C18H), 1.35 (3H,s C19H), 4.00 (1H,m 11βH), 5.74 (1H,s C4H). Also identified was 7β-hydroxyandrost-4-ene-3,17-dione [70] (0.32 g), m.p.

218-220°C (lit.[75] 220.5-222.5°C). The $^{1}H$ NMR spectrum included signals at δ 0.94 (3H,s C18H), 1.24 (3H,s C19H), 3.52 (1H,m 7αH), 5.78 (1H,s C4H). Mass Spec. M/e (%): 302 (100), 287 (16), 284 (42), 269 (31). $^{13}C$ NMR data are presented in Table 1.

Androst-4,6-diene-3,17-dione

Incubation of 1.7 g of this substrate for control purposes with R. arrhizus for 21 hours provided 1.8 g of crude extract. Tlc showed the extract to be a complex mixture of products. Attempts were made to separate the individual components using preparative tlc plates (silica gel). The solvent mixtures employed in the development of the plates included 25% acetone/hexane, 10% acetone/benzene and 5% methanol/benzene. Only three components were isolated in a pure state. They included 6α,7α-epoxyandrost-4-ene-3,17-dione [62] (0.15 g) identical with chemically prepared material, 6β,7β-epoxyandrost-4-ene-3,17-dione [71] (0.23 g), m.p. 210-213°C. The $^{1}H$ NMR spectrum included signals at δ 0.96 (3H,s C18H), 1.24 (3H,s C19H), 3.45 (2H,s 6αH,7αH), 6.17 (1H,s C4H). Mass spec. $M^{+}$ found 300.175; calculated for $C_{19}H_{24}O_3$ 300.172. M/e(%): 300 (9), 285 (9), 185 (48), 149 (56), 71 (100). $R_f$ values: 0.80 (benzene:ethyl acetate:acetone 3:3.5:3.5), 0.81 (benzene:acetone 3:7), 0.61 (acetone:hexane 8:2). $^{13}C$ NMR data are presented in Table 1. and 17β-hydroxyandrost-4,6-diene-3,16-dione [72] (0.16 g), m.p. 178-180°C. The $^{1}H$ NMR spectrum included signals at δ 0.83 (3H,s C18), 1.15 (3H,s C19), 3.81 (1H,s 17αH), 5.71 (1H,s C4H), 6.08 (2H,m C6H,C7H). Mass spec. $M^{+}$ found 300.162, calculated for $C_{19}H_{24}O_3$ 300.172, M/e (%): 300 (16), 282 (8), 267 (5), 228 (23), 91 (100). $^{13}C$ NMR data are presented in

Table 1. Analysis: (found C 75.00%, H 8.04%; $C_{19}H_{24}O_3$ requires C 75.96%, H 8.06%).

7α-Hydroxyandrost-4-ene-3,17-dione [63]

Incubation of 0.2 g of this substrate for 50 hours with R. arrhizus provided 0.25 g of crude extract. Tlc showed only the starting material was present. Recrystallization of the crude residue from acetone gave 0.18 g of the substrate.

Table 1. $^{13}C$ chemical shifts of steroidal products (values in ppm relative to TMS = 0, solvent: $CDCl_3$).

| C-# | [46] | [47] | [48] | [49] | [51] | [52] | [53] |
|---|---|---|---|---|---|---|---|
| 1 | 35.7 | 35.7 | 35.7 | 37.3 | 35.5 | 35.3 | 35.8 |
| 2 | 33.9 | 33.9 | 33.9 | 34.2 | 33.8 | 33.7 | 33.9 |
| 3 | 199.2 | 199.3 | 199.2 | 199.1 | 197.6 | 198.9 | 199.9 |
| 4 | 124.1 | 124.1 | 124.1 | 124.1 | 124.9 | 123.1 | 124.0 |
| 5 | 170.6 | 170.6 | 170.5 | 169.5 | 166.8 | 170.6 | 171.3 |
| 6 | 32.7 | 32.7 | 32.7 | 32.8 | 29.8 | 32.1 | 32.9 |
| 7 | 31.8 | 31.9 | 31.8 | 32.1 | 27.6 | 31.7 | 32.0 |
| 8 | 35.6 | 35.6 | 35.7 | 31.1 | 35.5 | 31.1 | 35.8 |
| 9 | 53.6 | 53.6 | 53.6 | 145.2 | 87.7, 89.1 | 55.6, 57.0 | 53.7 |
| 10 | 38.5 | 38.4 | 38.5 | 41.0 | 45.7 | 38.9 | 38.6 |
| 11 | 20.9 | 20.9 | 21.0 | 118.4 | 86.4, 98.5 | 83.9, 95.6 | 20.9 |
| 12 | 38.5 | 38.5 | 38.5 | 40.7 | 39.3, 40.1 | 43.8, 45.1 | 37.9 |
| 13 | 44.6 | 44.9 | 44.7 | 42.3 | 42.5 | 42.6, 42.7 | 44.0 |
| 14 | 56.0 | 56.2 | 56.0 | 52.9 | 50.6 | 56.6 | 55.1 |
| 15 | 24.4 | 24.5 | 24.4 | 26.0 | 23.5 | 24.2 | 24.4 |
| 16 | 23.5 | 22.5 | 23.7 | 25.4 | 22.6 | 22.7 | 23.4 |
| 17 | 59.9 | 57.7 | 60.2 | 63.4 | 63.1 | 63.6 | 55.5 |
| 18 | 13.5 | 13.6 | 13.6 | 13.0 | 14.9, 15.4 | 14.7, 15.2 | 13.3 |
| 19 | 17.3 | 17.4 | 17.4 | 23.0 | 22.2, 23.1 | 20.1, 20.8 | 17.4 |
| 20 | 202.1 | 206.0, 207.2 | 201.8 | 209.0 | 208.2 | 208.4 | 179.8 |
| 21 | 49.4 | 79.2, 91.6 | 35.7 | 33.8 | 33.8 | 32.1 | |
| 22 | | | | | | | |

Table 1. (continued)

| C-# | [55] | [60] | [62] | [63] | [70] | [71] | [72] |
|---|---|---|---|---|---|---|---|
| 1 | 35.6 | 33.9 | 34.0 | 35.4 | 33.9 | 34.0 | 33.6 |
| 2 | 33.9 | 33.9 | 33.9 | 33.9 | 31.3 | 34.9 | 33.8 |
| 3 | 199.5 | 199.4 | 199.2 | 199.3 | 199.4 | 198.0 | 199.5 |
| 4 | 124.0 | 124.3 | 131.5 | 127.0 | 124.9 | 129.7 | 124.3 |
| 5 | 171.1 | 162.9 | 161.8 | 168.0 | 167.4 | 162.1 | 162.9 |
| 6 | 32.8 | 128.8 | 52.6 | 39.4 | 38.3 | 55.6 | 128.9 |
| 7 | 31.9 | 138.5 | 53.5 | 67.0 | 74.2 | 57.7 | 138.8 |
| 8 | 35.6 | 37.1 | 35.6 | 41.1 | 36.0 | 36.1 | 36.1 |
| 9 | 53.7 | 48.8 | 46.8 | 45.3 | 50.6 | 51.6 | 46.8 |
| 10 | 38.7 | 36.1 | 34.3 | 38.6 | 38.1 | 36.6 | 35.1 |
| 11 | 21.0 | 20.1 | 19.2 | 20.2 | 20.4 | 20.5 | 20.1 |
| 12 | 38.6 | 31.3 | 31.1 | 31.0 | 31.2 | 31.0 | 36.3 |
| 13 | 44.1 | 48.3 | 47.8 | 37.3 | 48.1 | 48.3 | 43.3 |
| 14 | 56.1 | 50.8 | 40.9 | 45.6 | 50.8 | 47.9 | 50.7 |
| 15 | 24.4 | 21.5 | 21.4 | 21.3 | 24.9 | 21.5 | 36.1 |
| 16 | 23.1 | 35.6 | 35.6 | 35.8 | 35.6 | 35.5 | 216.1 |
| 17 | 62.5 | 219.5 | 219.5 | 220.8 | 221.0 | 219.1 | 85.9 |
| 18 | 13.5 | 13.7 | 13.7 | 13.5 | 13.9 | 13.7 | 11.3 |
| 19 | 17.4 | 17.3 | 17.3 | 17.0 | 17.4 | 16.8 | 16.3 |
| 20 | 211.9 | | | | | | |
| 21 | 33.9 | | | | | | |
| 22 | 7.7 | | | | | | |

# RESULTS AND DISCUSSION

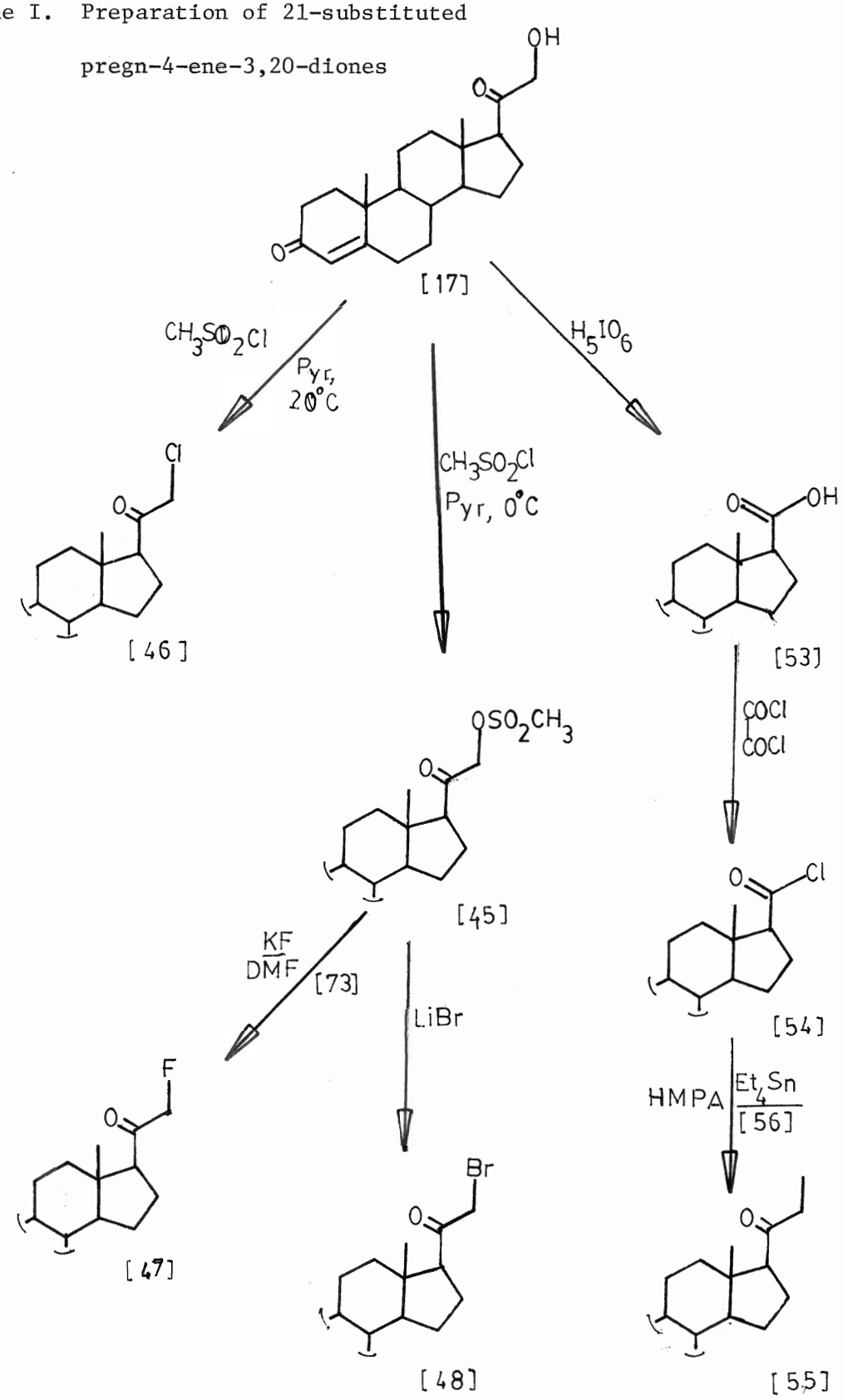
## Preparation of steroidal substrates

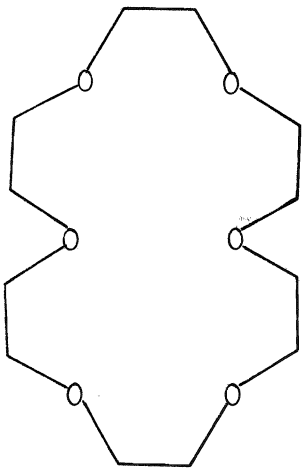
In this study, attempts were made to halogenate three positions of the steroid nucleus, namely at carbon 21, 11 and 7. Due to steric difficulties and the different reactivity of each position, it was necessary to implement different synthetic strategies in each case. In all three cases, the steroid skeleton possessed the $\Delta^4$-3-keto function. The products from the reactions were identified by comparison of their physical constants with those in the literature and by spectroscopic examination. $^1$H NMR, $^{13}$C NMR and mass spectrometry were especially useful for fast identification of the various products.

The procedure followed in the preparation of the 21-halogenated steroids was quite straightforward (Scheme 1). Yields were moderate (in the case of 21-fluoropregn-4-ene-3,20-dione [47]) to good for the 21-chloro and bromo analogues. During the preparation of [47], the crown ether [73] was added to chelate potassium ions, thus providing a greater concentration of "free" fluoride ions to participate in the nucleophilic substitution. The use of the crown ether significantly improved the rate of formation and the yield of the 21-fluorinated product [47].[49]

During the preparation of 21-methanesulfonyloxypregn-4-ene-3,20-dione [45], it was observed that when the temperature of the reaction was allowed to rise above 0°C, two products were obtained, whereas when

Scheme I. Preparation of 21-substituted pregn-4-ene-3,20-diones





[73]

the temperature was kept below 0°C, only [45] was isolated, which was identical to one of the two products obtained above. Analysis of the second product proved it to be 21-chloropregn-4-ene-3,20-dione [46]. When the reaction was repeated at ambient temperature, only [46] was obtained in high yield. The chlorinated product most likely resulted from the internal displacement of the mesylate group by the chloride ion (Fig. 9).

The procedure followed in the preparation of 21-methylpregn-4-ene-3,20-dione [55] is not original,[66] but to the author's knowledge, no previous reports have been made applying this relatively new procedure to the preparation of steroidal ketones. As outlined in the experimental section, the final reaction in the preparation of [55] involved the reaction of tetraethyltin and the acid chloride [54] in the presence of the catalyst [56]. At first glance, this reaction appears to be a simple transfer of ethyl groups and chloride ions to the steroid and the tin compound respectively. However, a potentially more complex mechanism involving the catalyst [56] has been suggested (Fig. 10).[66]

The proposed mechanism suggests that the active catalyst bis(triphenylphosphine)palladium(0) [75] is generated from [56] by a series of double exchanges and reductive eliminations. The acid chloride [54] then oxidatively adds to the active catalyst [75] generating a palladium(II) complex which undergoes metathesis with the tin compound and reductively eliminates the product ketone.

The yield of 21-methylpregn-4-ene-3,20-dione [55] was moderate at 40%, but the conditions for optimum yield were not fully investigated.

Figure 9. Suggested mechanism for the preparation of 21-chloro-pregn-4-ene-3,20-dione

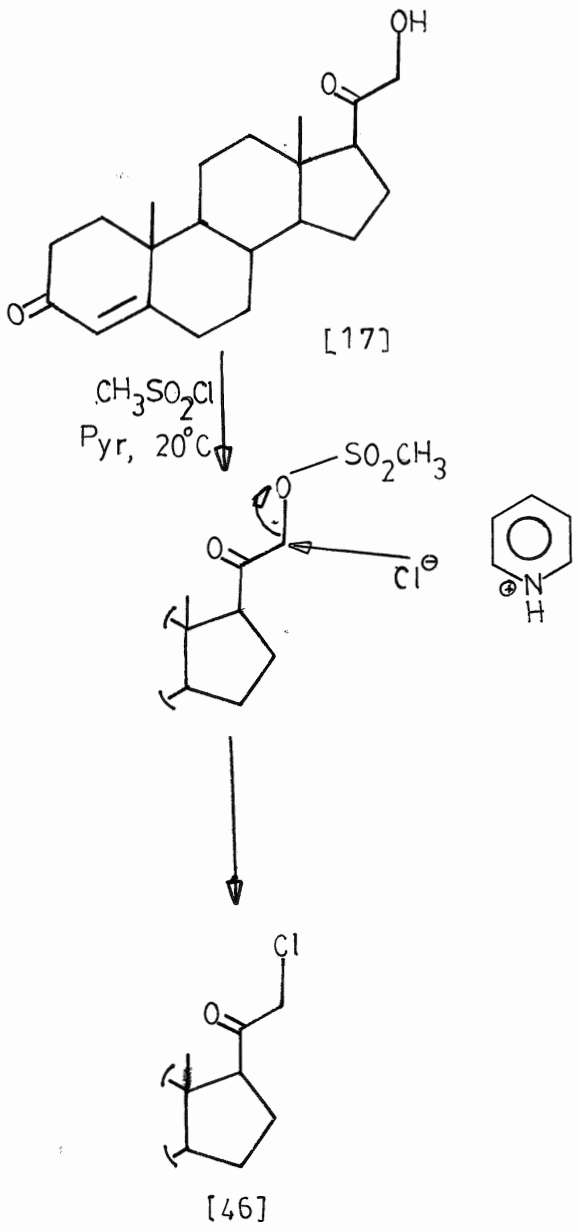
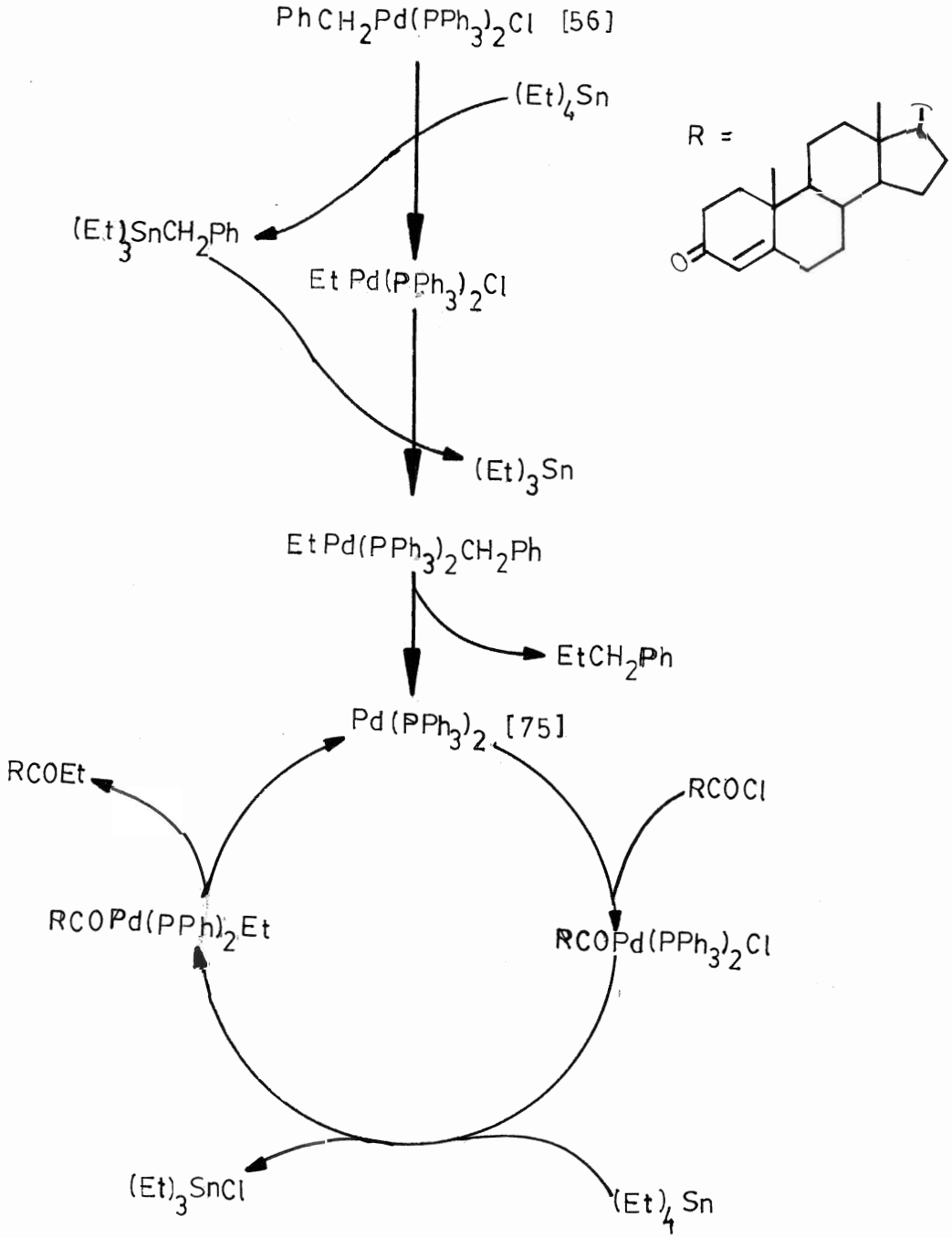


Figure 10. Suggested mechanism for the preparation of 21-methyl-pregn-4-ene-3,20-dione

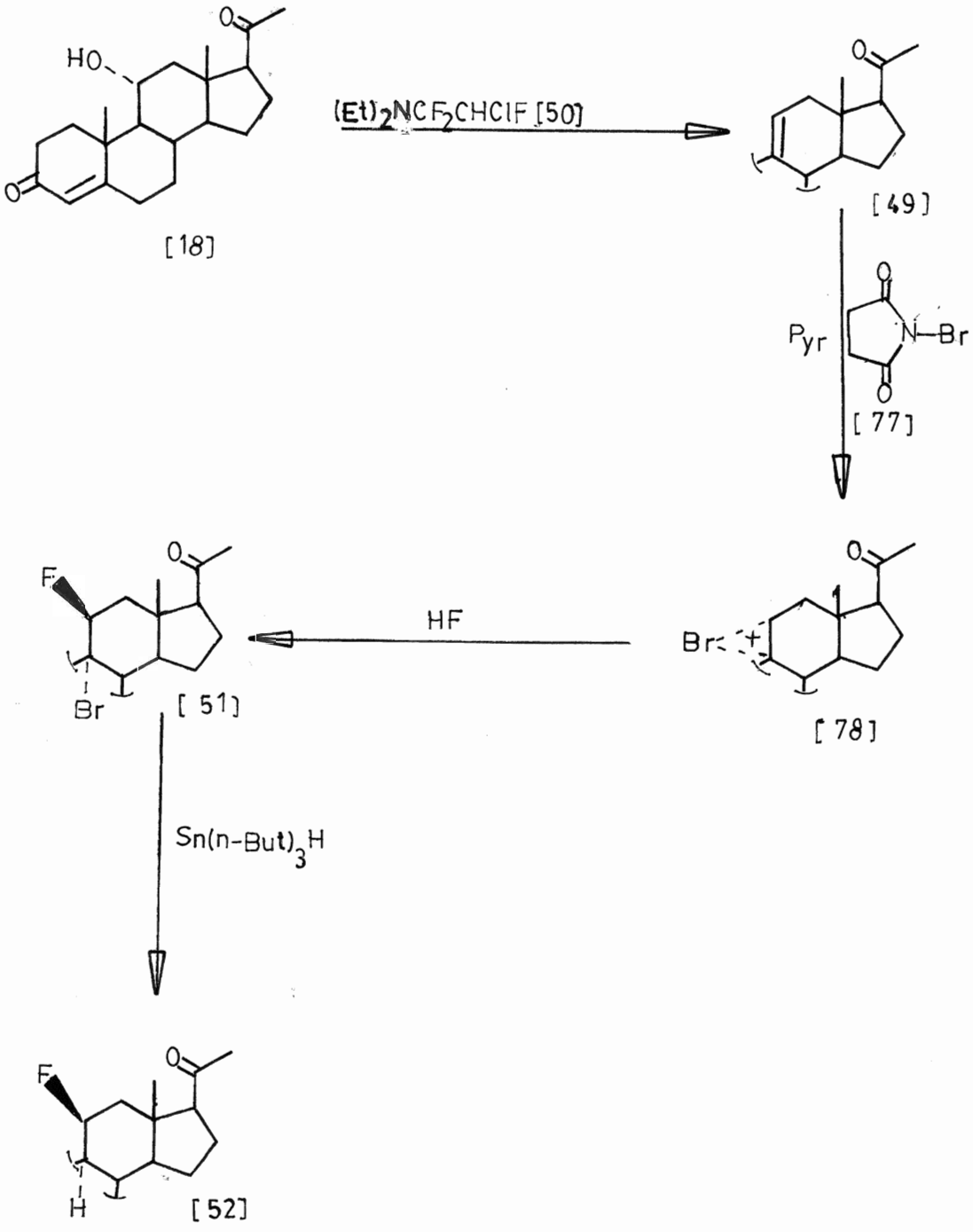


From preliminary studies, the rate of the reaction appears to be temperature dependent, since at 80°C, the reaction was complete in about 50 percent of the time required when performed at 65°C. The catalyst appears to be unstable over extended periods of time since the desired product was not obtained when catalyst prepared two weeks earlier was used. The procedure is relatively simple when compared to previously reported procedures for the preparation of compounds similar in structure to the one under consideration. The possibility exists that this procedure could be of value in further lengthening of the progesterone side chain.

The preparation of 11β-fluoropregn-4-ene-3,20-dione [52] was also accomplished through the use of literature procedures.[59,61] Although other procedures have been described[50,62] for the preparation of 11β-fluorinated steroids, all attempts to repeat them were unsuccessful. The successful procedure involved the trans addition of bromine and fluorine to pregn-4,9(11)-diene-3,20-dione [49] using the reagents, N-bromosuccinimide and 70% HF/pyridine mixture. The use of the 70% HF/pyridine reagent eliminated obvious difficulties associated with the use of anhydrous hydrofluoric acid. In the reaction, the conjugate acid of N-bromosuccinimide [77] serves as a source of electrophilic bromine which reacts with the olefin from the less hindered α-side to form the bridged ion [78]. This ion in turn reacts stereospecifically with fluoride to form the trans bromofluoro- compound [51] (Scheme II).[59,92]

Pregn-4,9(11)-dione-3,20-dione [49) was prepared in high yield from 11α-hydroxypregn-4-ene-3,20-dione [18] using 2-chloro-1,1,2-trifluoro-

Scheme II. Preparation of 11β-fluoropregn-4-ene-3,20-dione



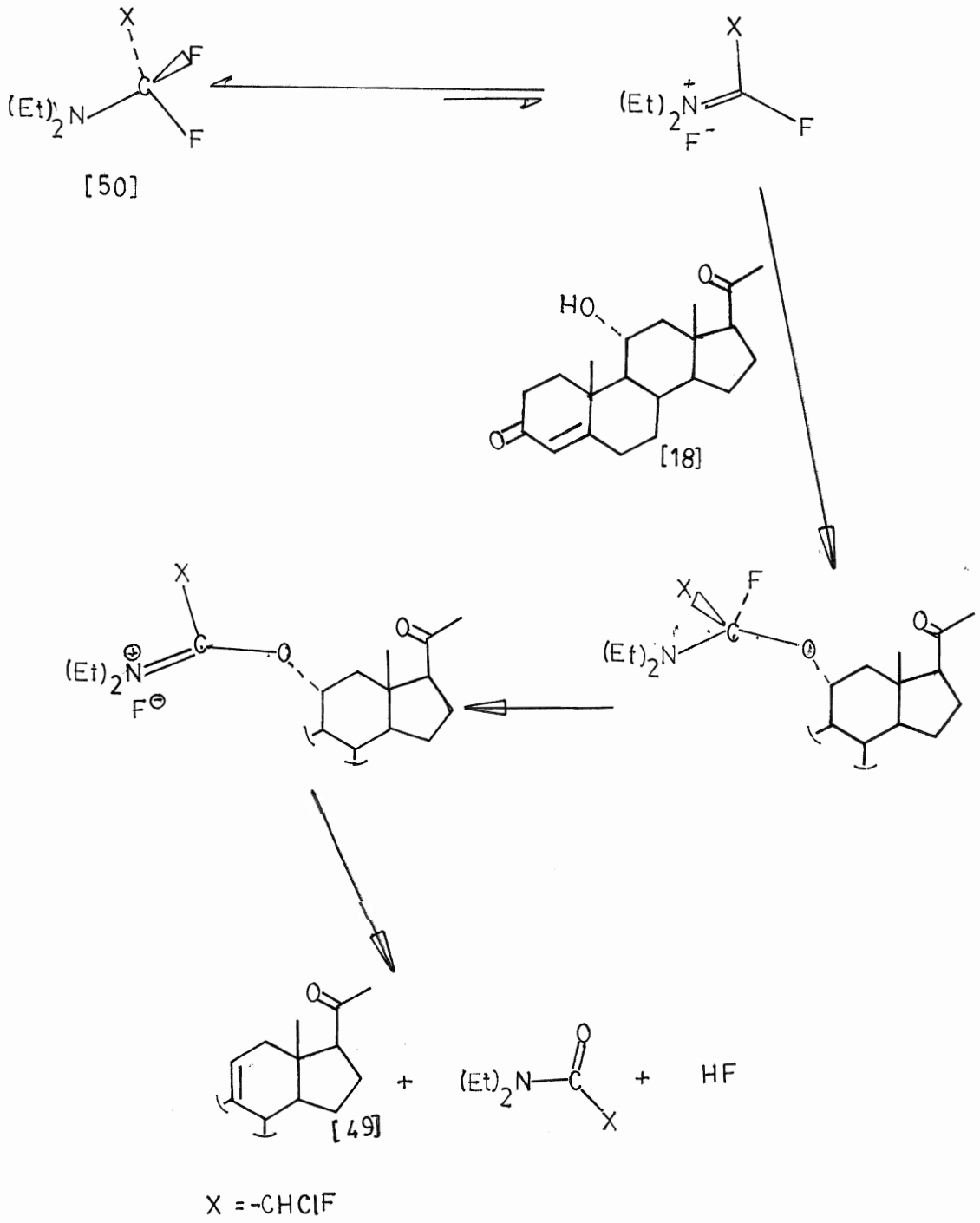
triethylamine [50]. Our first intention was to use [50] as a fluorinating reagent since this reagent was reported[50,62] to react with [18] to give the 11β-fluoro analogues [52]. In all attempts, no fluorinated steroids were isolated even when the reaction was performed below -50°C. The mechanism involved in the fluorination of steroidal alcohols by [50] is not fully understood, but is believed to be quite complex, since the types of products obtained depends not only on the conditions of the reaction, but also on the position of the hydroxy group in the steroid skeleton. The reaction of [50] with 11α-hydroxypregn-4-ene-3,20-dione [18] appears to involve very unstable intermediates which readily eliminate due to the high degree of steric hindrance at carbon-11 (Scheme III).[62]

The bromine atom was then reductively eliminated from 9α-bromo-11β-fluoropregn-4-ene-3,17-dione[51] using tri-n-butyltin hydride to give 11β-fluoropregn-4-ene-3,20-dione [52] (Scheme II). The tributyltin hydride reagent is reported to reduce tertiary halides leaving primary and secondary halides unaffected.[74]

An interesting aspect of both [51] and [52] is the long range coupling of the fluorine atom to carbons and protons 4 and 5 bonds away respectively. There appears to exist some controversy over whether the coupling occurs through space or through the bonds adjoining the nuclei.[55,93,94]

In an earlier study[93] electronegative groups were placed at various positions to observe their effect on the magnitude of the coupling constants (J) between the 6β-fluorine and the 18- and 19-methyl protons of several steroids. It was reported that the introduction of a chlorine

Scheme III. Mechanism for the dehydration of 11α-hydroxypregn-4-ene-3,20-dione

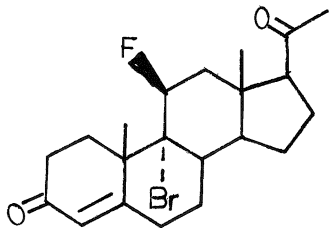


atom at the 9α-position of the 11-fluorinated steroid [79] did not significantly affect the magnitude of $J_{18H,11\beta F}$, whereas there was a substantial increase in the $J_{19H,11\beta F}$ value. This finding was used to vaguely support a through space mechanism based on the assumption that the 9α-chlorine causes perturbation of the 19-methyl proton orbitals, but is too distant from the 18 protons to bring about comparable changes.

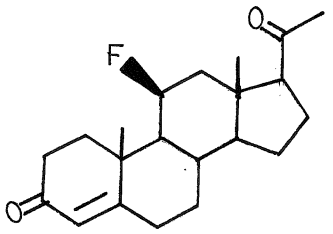
In the present study, introduction of a 9α-bromine in [51] appears to have no significant effect on the magnitude of $J_{19H,11\beta F}$ or $J_{18H,11\beta F}$ (see Table 2). Our results would appear to be more significant since in the earlier study, the J values were calculated from structurally different compounds, [79] and [80].

Further insights into the mechanism of these long range couplings were gained through examination of the $^{13}C$ NMR spectra of [51] and [52]. Comparison of the $J_{13C,11\beta F}$ values of the nuclei in the chain from both C18 and C19 to 11βF in [51] and [52] shows that the introduction of the bromine atom at the 9α position again does not significantly affect the magnitude of either $J_{18C^{13},11\beta F}$ or $J_{19C^{13},11\beta F}$ although the bromine atom appears to have distorted the B and C rings in such a manner that there is a slight increase in the magnitude of $J_{19C^{13},11\beta F}$ and a decrease in the magnitude of $J_{12C^{13},11\beta F}$ (see Table 3). The fact that the $J_{C^{13}-11\beta F}$ decreases sharply with increasing distance, and then increases for $J_{18C^{13},11\beta F}$ and $J_{19C^{13},11\beta F}$ is strong evidence supporting a through space coupling mechanism.

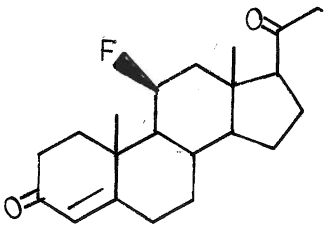
Attempts were made to prepare the 11-chlorinated analogue using the procedure just outlined above. Thus the trans addition of bromine and



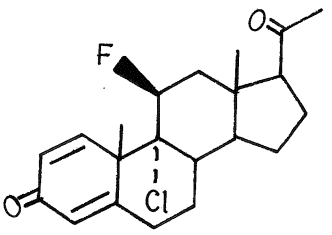

[51]




[52]




[79]




[80]

Table 2. $J_{18H,11\beta F}$ and $J_{19H,11\beta F}$ values (Hz) for [51] and [52].

| | [51] | [52] |
|---|---|---|
| $J_{18H,11\beta F}$ | 2.93 | 2.74 |
| $J_{19H,11\beta F}$ | 4.03 | 4.03 |

Table 3. $J_{C^{13},11\beta F}$ values (Hz) for [51] and [52].

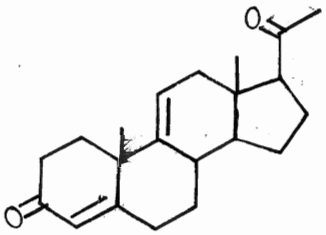
| Carbon # | [51] | [52] |
|---|---|---|
| 19 | 13.77 | 9.18 |
| 10 | 0.0 | 0.0 |
| 9 | 20.20 | 22.20 |
| 11 | 142.18 | 176.13 |
| 12 | 20.20 | 19.19 |
| 13 | 0.0 | 1.84 |
| 18 | 7.34 | 6.42 |

chlorine to pregn-4,9(11)-diene-3,20-dione [49] using N-bromosuccinimide and anhydrous hydrochloric acid furnished the unstable 9α-bromo-11β-chloropregn-4-ene-3,20-dione [80]. Subsequent attempts to reductively eliminate the bromine atom again using the tin hydride reagent were unsuccessful. A recent report has claimed the preparation of 11β-chloropregn-4-ene-3,20-dione [81] in 4% yield by the treatment of 11α-hydroxypregn-4-ene-3,20-dione [18] with [50] and lithium chloride.[91]

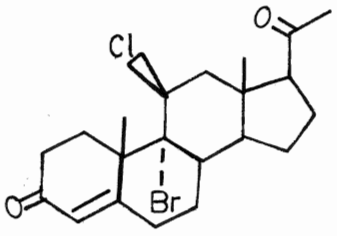
Attempts to halogenate the third position (carbon 7) of the steroid skeleton proved much more difficult than first anticipated. Numerous approaches were tried unsuccessfully to displace both the 7α and 7β hydroxy groups of androst-4-ene-3,17-dione [10] using a variety of reagents. Attempts to prepare sulfonate esters which could act as good leaving groups were also unsuccessful.

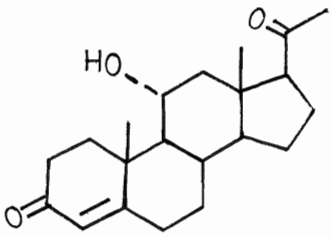
The difficulties encountered in the preparation of the $\Delta^4$-3-keto-7-halogenated steroids may be attributed to the instability of these analogues or alternatively to the stability of the $\Delta^{4,6}$-3-keto function. In many instances, the product isolated after attempted nucleophilic displacement of the 7-hydroxy group or from attempts to prepare sulfonate esters was the 6-dehydro-steroid [60].

Since both the 7α and 7β hydroxy steroids are stable, it is reasonable to postulate that in some instances, nucleophilic displacement or sulfonate ester formation did occur, but these products quickly decomposed to [60] through the elimination of the appropriate hydrohalic acid or sulfonic acid (Fig. 11).



[49]

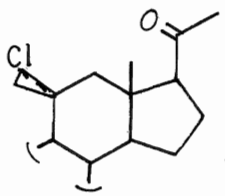




[18]

$(Et)_2NCHClF_2$ [50]

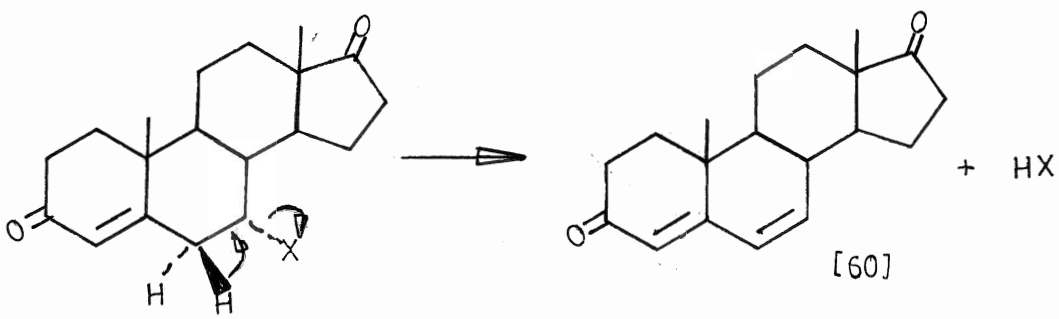
LiBr



4%

[81]

Figure 11. Proposed mechanism for the elimination of 7α substituents from $\Delta^4$-3-keto steroids




X = Br, Cl, $CH_3SO_3^-$, $CH_3PhSO_3^-$

Towards the end of this study, a patented paper[88] was obtained which reported procedures for the preparation of $\Delta^4$-3-keto-7-halogenated steroids. These compounds were reported to be unstable at room temperature since they spontaneously eliminated hydrohalic acid giving the $\Delta^{4,6}$-3-keto steroid. This elimination is quite rapid in a basic environment.

Incubations

Table 4 shows the various steroids used as substrates and the results of incubating them with the four fungi listed. The new as well as known compounds were identified mainly by spectroscopic examination. Among the spectrometric methods for locating oxygenated substituents (particularly hydroxyl groups) in the steroid nucleus was examination of the $^{1}$H-NMR signal of the 18 and 19 protons. The large volume of reference data[95,96] and the assumption that substituents present in the steroid nucleus contribute a fairly constant increment to the chemical shift of the 18 and 19 protons and that these increments are additive provided the basis for using this technique.

The position and form of the CHOH resonance also provided valuable information regarding the position of hydroxylation and some times about the configuration of the hydroxyl group. Mass spectrometry was generally employed to confirm the number of oxygens introduced into the substrates by a particular fungus.

Incubation with Aspergillus niger ATCC 9142

It has been reported that the replacement of a C-21 hydrogen of pregn-4-ene-3,20-dione [11] by a fluorine atom increases the progestational activity of this new analogue by a factor of 2-4 over [11].[16] The same substitution with chlorine, bromine or a methyl group also gave progestational activity, but much lower than [11].[67,97,98]

The suggestion has been made that halogen substitution into the steroid nucleus sometimes retards biochemical degradation of these

Table 4 Results from incubation of the substrates listed with the four fungi listed.*

| Substrate | Fungus | Substrate recovered (%) | Products (%) |
|---|---|---|---|
| Pregn-4-ene-3,20-dione | Aspergillus niger ATCC 9142 | 25 | 21-hydroxypregn-4-ene-3,20-dione (25) |
| 21-Fluoropregn-4-ene-3,20-dione | Aspergillus niger ATCC 9142 | 44 | none identified |
| 21-Chloropregn-4-ene-3,20-dione | Aspergillus niger ATCC 9142 | 60 | none identified |
| 21-Bromopregn-4-ene-3,20-dione | Aspergillus niger ATCC 9142 | 62 | none identified |
| 21-Methylpregn-4-ene-3,20-dione | Aspergillus niger ATCC 9142 | 80 | none identified |
| Pregn-4-ene-3,20-dione | Curvularia lunata NRRL 2380 | 30 | 11β-hydroxypregn-4-ene-3,20-dione (24)<br>14α-hydroxypregn-4-ene-3,20-dione (20)<br>11β,14α-dihydroxypregn-4-ene-3,20-dione (10) |
| 11β-Fluoropregn-4-ene-3,20-dione | Curvularia lunata NRRL 2380 | 29 | 11β-fluoro-14α-hydroxypregn-4-ene-3,20-dione (17)<br>11β-fluoro-14α,21-dihydroxypregn-4-ene-3,20-dione (7) |
| 11β-Fluoropregn-4-ene-3,20-dione | Rhizopus stolonifer ATCC 6227b | 54 | Pregn-4-ene-3,11,20-trione (33) |
| 11β-Hydroxypregn-4-ene-3,20-dione | Rhizopus stolonifer ATCC 6227b | 0 | Pregn-4-ene-3,11,20-trione (85) |
| Androst-4-ene-3,17-dione | Rhizopus stolonifer ATCC 6227b | 21 | 6β-hydroxyandrost-4-ene-3,17-dione (36)<br>7β-hydroxyandrost-4-ene-3,17-dione (8)<br>11α-hydroxyandrost-4-ene-3,17-dione (5) |
| Androst-4,6-diene-3,17-dione | Rhizopus arrhizus ATCC 11145 | 10 | 6β,7β-epoxyandrost-4-ene-3,17-dione (14)<br>6α,7α-epoxyandrost-4-ene-3,17-dione (8)<br>17β-hydroxyandrost-4,6-diene-3,16-dione (9) |
| 7α-Hydroxyandrost-4-ene-3,17-dione | Rhizopus arrhizus ATCC 11145 | 90 | none identified |

* For conditions used, see experimental section.

analogues and thus increases the duration of the physiological effects. It was therefore of interest to observe the effect halogen substitution at the C-21 position had on hydroxylation at that position using the fungus Aspergillus niger, a known C-21-hydroxylator.[27,99] The results from the various incubations are listed in Table 4.

The incubation of 21-fluoropregn-4-ene-3,20-dione [47] with A. niger furnished mostly unreacted substrate, but also a small quantity of an unidentified product having a molecular weight of 348 (determined by mass spectrometry). This product which is 16 mass units heavier than the substrate suggested that hydroxylation had occurred at a site other than the C-21 position. This is assumed since, if hydroxylation had occurred at the 21-position, the resulting geminal halohydrin would be expected to be unstable and not amenable to isolation.

The incubation of the 21-chloro- [46] and 21-bromo [48] analogues furnished only unreacted substrates. Only about 60 percent of the substrate was recovered in each case. This low recovery of substrate may be attributed to difficulties encountered during the extraction of the mycelia. Alternatively, the low recovery could also stem from initial 9α-hydroxylation since this process is known[100,101] to be important in microbiological degradation of steroids. The incubation of 21-methyl-pregn-4-ene-3,20-dione [55] gave starting material largely unchanged.

The results obtained above are consistent with previous studies which have shown that the presence of an halogen at a favoured site of hydroxylation generally blocks hydroxylation at that site.[47,48] Obviously, the C21-methyl group has the same effect. In previous studies,

the halogens were located at rigid positions while in the case of the 21-halogenated substrates, the methyl hydrogens have more freedom of rotation and it was expected that the presence of the halogens at the less rigid position would have less effect on hydroxylation. Therefore, it appears that the three hydrogens at C-21 are essential for C21-hydroxylation to proceed using the fungus A. niger.

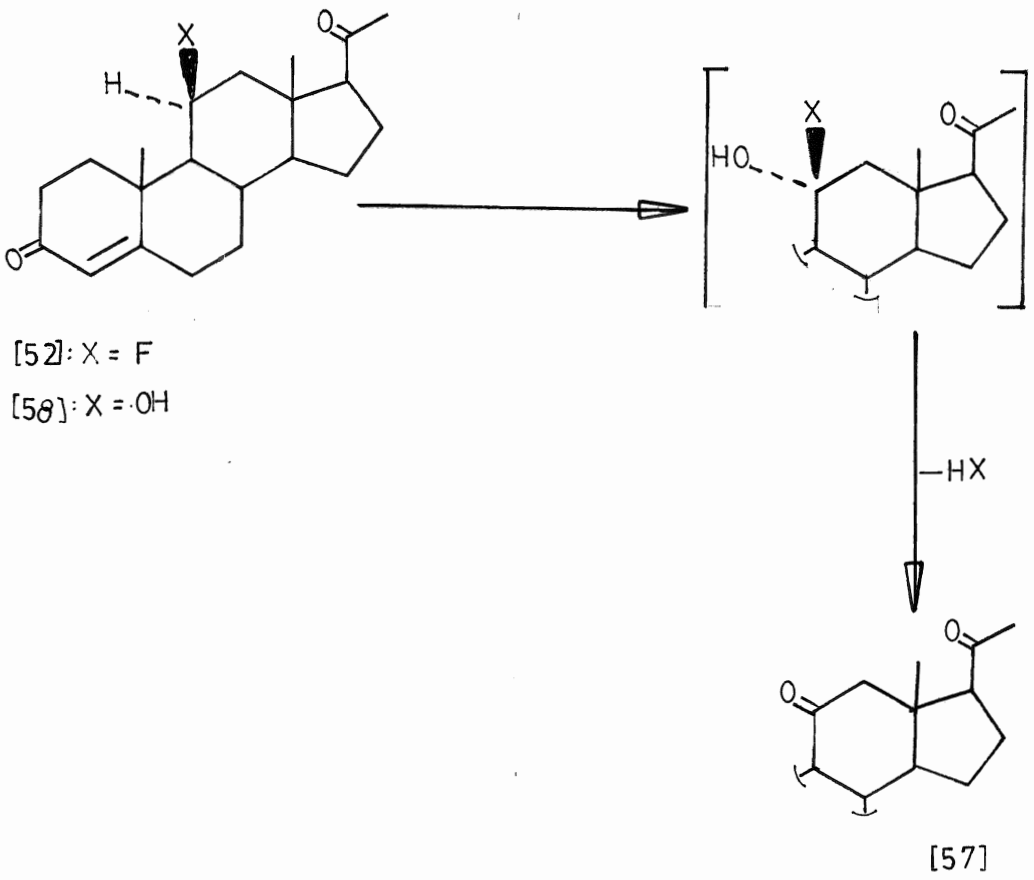
Incubation with Curvularia lunata and Rhizopus stolonifer

Curvularia lunata is known to hydroxylate the steroid nucleus at several positions, a favoured one being the 11β-position of C-21 steroids having the $\Delta^4$-3-keto function. In contrast, R. stolonifer hydroxylates the same steroids predominantly at the 11α-position. When pregn-4-ene-3,20-dione was incubated with C. lunata, three products were identified. They included the 11β- and 14α- monohydroxylated, and 11β,14α-dihydroxylated products. The incubation of the same substrate with R. stolonifer is reported to provide only the 11α-hydroxylated product.

It was of interest to compare the effects of a fluorine atom at a favoured site of hydroxylation (in case of C. lunata) and the effects the fluorine atom at a position geminal to that favoured for hydroxylation (in case of R. stolonifer) had on the metabolism of such a steroid.

The incubation of 11β-fluoropregn-4-ene-3,20-dione [52] with both fungi provide significantly different products (Table 4). Whereas C. lunata provided products hydroxylated at sites remote from the fluorine atom, R. stolonifer provided only the keto product [57] which in all probability resulted from initial hydroxylation at the 11α-position of [52] followed by loss of HF from the unstable halohydrin. In both cases

Figure 12. Hydroxylation of 11β-substituted pregn-4-ene-3,20-dione by Rhizopus stolonifer

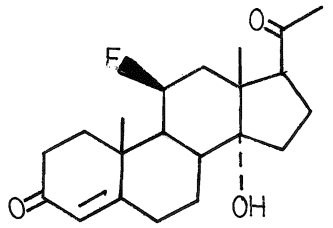


the presence of the fluorine slows the apparent rate of hydroxylation of [52].

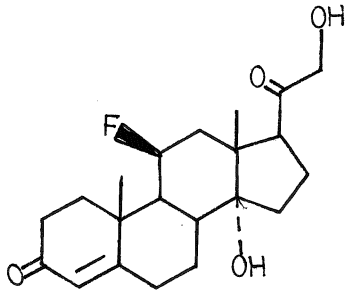
The incubation of 11β-hydroxypregn-4-ene-3,20-dione [58] with R. stolonifer also provided the trione [57]. However, in this case the conversion was quite fast. Within 36 hours [58] was completely converted to [57]. Again it is reasonable to postulate that the 11-keto group resulted from the loss of water after initial 11α-hydroxylation of the substrate (see Fig. 12). However, it is also possible that the 11β-hydroxy group was simply oxidized by an alcohol dehydrogenase. The results obtained with the previous incubation with [52] suggest that the former pathway is more plausible.

The products obtained from the incubation of [52] with C. lunata included the 14α-monohydroxylated [66] and 14α,21-dihydroxylated [67] metabolites. The fluorine atom appears to have directed hydroxylation from the 11β-position to the 21-position. C-21-hydroxylation by this strain of C. lunata has not been reported previously.

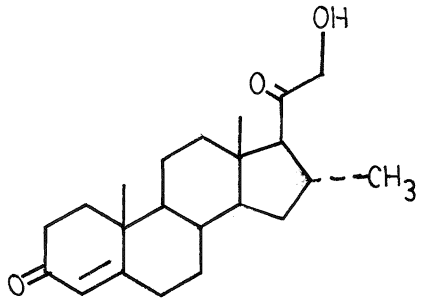
Fluorine atoms at sites other than those favoured for hydroxylation also have an effect on the positions in the substrate that are hydroxylated. In one report,[102] the introduction of a fluorine at the 6α position of 21-hydroxy-16α-methylpregn-4-ene-3,20-dione [83] provided products monohydroxylated at the 9α, 11β and 14α positions whereas the non-fluorinated substrate [82] gave products monohydroxylated at the 7α, 11β and 14α positions when incubated with C. lunata. Obviously, the fluorine atom at a site vicinal to one favoured for hydroxylation also blocks hydroxylation at that site.



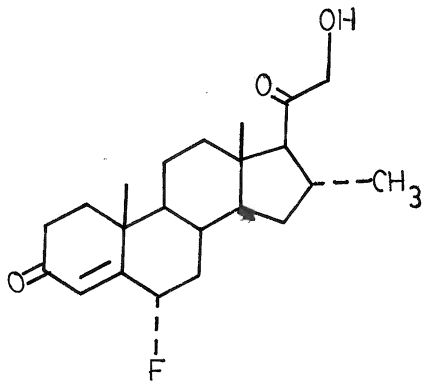

[66]




[67]




[82]




[83]

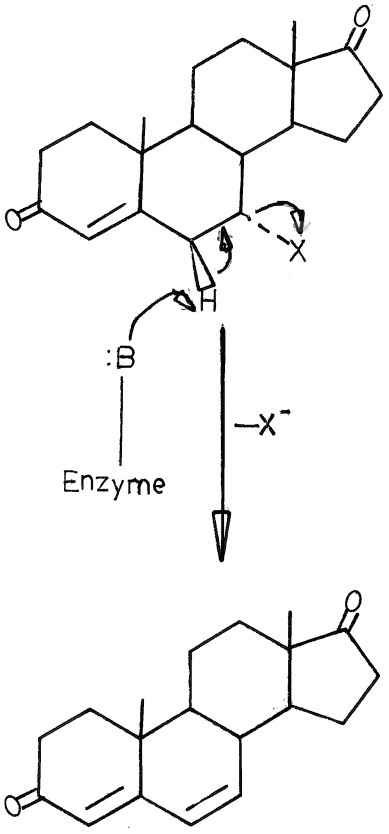
Therefore while with C. lunata, the site of hydroxylation depends on the positions of the substituents in the substrate, R. stolonifer shows a predilection for hydroxylation at the 11α-position. The present findings are consistent with earlier studies with saturated steroids which have shown some fungi to be site specific and others to be substituent directed.[36-38]

At present it is not clear whether a single hydroxylase or a general hydroxylase system is at work in the production of the many different products that are obtained depending on the structure of the substrate in the case of C. lunata and many other fungi. That the enzymes responsible for hydroxylation of many positions of the steroid nucleus are inducible has led at least one investigator[103] to suggest that a generalized hydroxylase system is at work during the hydroxylation of 19-norsteroids by C. lunata. It is clear however that C. lunata, unlike A. niger possesses an abundance of hydroxylases whether in a complex or acting independently that are able to hydroxylate the various positions of the steroid skeleton.

Incubation with Rhizopus arrhizus

This fungus is well documented as a 6β-hydroxylator of steroids having the $\Delta^4$-3-keto function. In previous studies[28,32] in this laboratory, strong evidence was obtained showing the participation of an enolic intermediate during 6β-hydroxylation of androst-4-ene-3,17-dione [10] by R. arrhizus (see Fig. 4). It was therefore postulated that if an enzyme was involved in the enol formation during 6β-hydroxylation, then

Figure 13. Mechanism involved in the formation of the proposed "suicide" substrate.



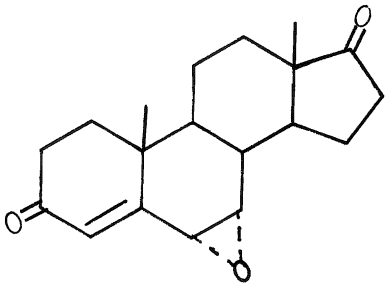
by the placement of a "good" leaving group at the 7α-position of [10], an intermediate, 6-dehydro steroid may result which the fungus cannot further metabolize. This so-called "suicide" substrate, if subsequently isolated, would provide further evidence supporting an enolic intermediate during 6β-hydroxylation.

A 7α substituent would be expected to eliminate faster than a 7β substituent since during 6β hydroxylation, the enolic intermediate results from the loss of the 6β axial proton. The 6β-proton is trans diaxial to a 7α substituent, which thus has the correct stereochemistry for elimination as shown in Figure 13.

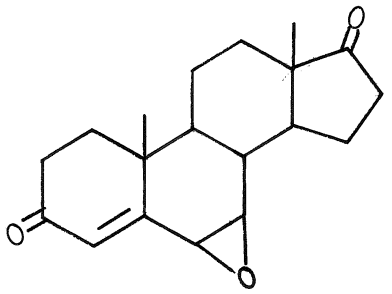
The compounds 7α-chloro- and 7α-bromoandrost-4-ene-3,17-dione were postulated as having the required properties to test the above hypothesis. Unfortunately, all attempts to prepare them were unsuccessful, most likely due to their instability as previously mentioned.

Although all attempts to prepare the 7α-halosteroids failed, the would be "suicide" substrate androst-4,6-diene-3,17-dione [60] was fermented with R. arrhizus for control purposes. The products obtained were quite unexpected. They included both 6β,7β- and 6α,7α-epoxyandrost-4-ene-3,17-dione ([62] and [71]), 17β-hydroxyandrost-4,6-diene-3,16-dione [72] plus at least two other unidentified products which were quite difficult to separate.

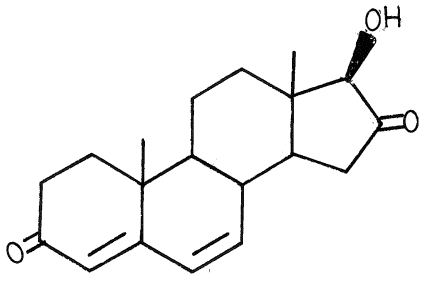
It is well documented, and was mentioned in the introduction, that the introduction of a double bond at $C_n$ of a substrate (where $C_n$ was the favoured site of hydroxylation) would result in epoxidation of the substrate at $C_n$ by the fungus only if the epoxide formed was axial. In this case, both the axial and the equatorial epoxides were obtained.



[62]



[71]



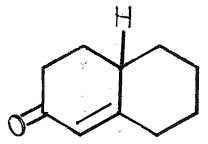
[72]

It is therefore apparent that during the oxidation the enzyme is able to approach from either the α or β face of the substrate to provide both the 6α,7α- and 6β,7β-epoxides. Thus, the isolation of [62] and [71] appears to be consistent with earlier findings that 6β-hydroxylation of $\Delta^4$-3-keto steroids by R. arrhizus is stereoelectronically controlled by the substrate and that the enzyme(s) involved are neither stereo- nor regiospecific.[28,32]

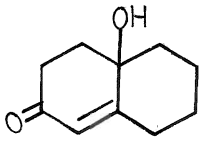
The earlier observations that the products [85], [86] and [87] are produced concurrently by R. arrhizus from [84] and also that [85], [86] and [87] are produced by peracid oxidation of [88] and [89] and that the incubation of the B-norsteroid [90] with R. arrhizus provided mostly the α-epoxide [91][104] does not support a mechanism wherein the regio- and stereospecificity of hydroxylation is determined solely by the three-dimensional relationship between the substrate and the enzyme's active site, although such a mechanism appears to be operative at saturated carbons remote from a keto group.[37,105] Structures similar to [88] and [89] are believed to be intermediates during enzymic oxidation of [84] to give [85], [86] and [87] by R. arrhizus.[76]

It is unlikely that an enolic intermediate is involved during the epoxidation at the C6-C7 bond. It is therefore possible that the enzyme is unable to differentiate between the structures [92] and [60].

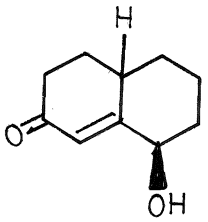
The third identified metabolite, 17β-hydroxy-4,6-diene-3,16-dione [72] is postulated as arising from 16 hydroxylation of the substrate [60] followed by rearrangement to give the more stable 17β-hydroxy-16-keto function (Fig. 14). The identity of this metabolite was ascertained by



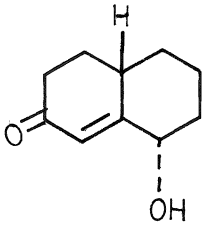

[84]



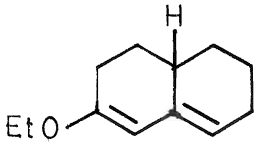

[85]



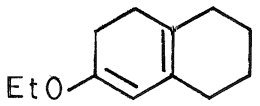

[86]



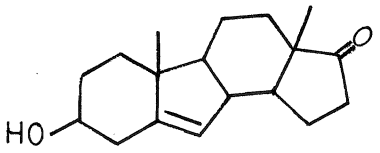

[87]



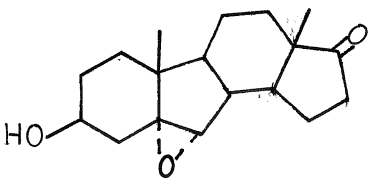

[88]



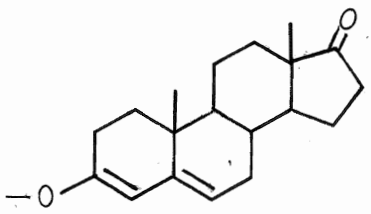

[89]



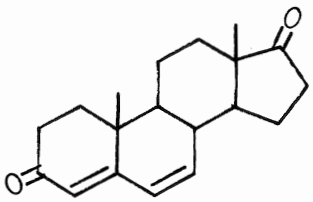

[90]




[91]

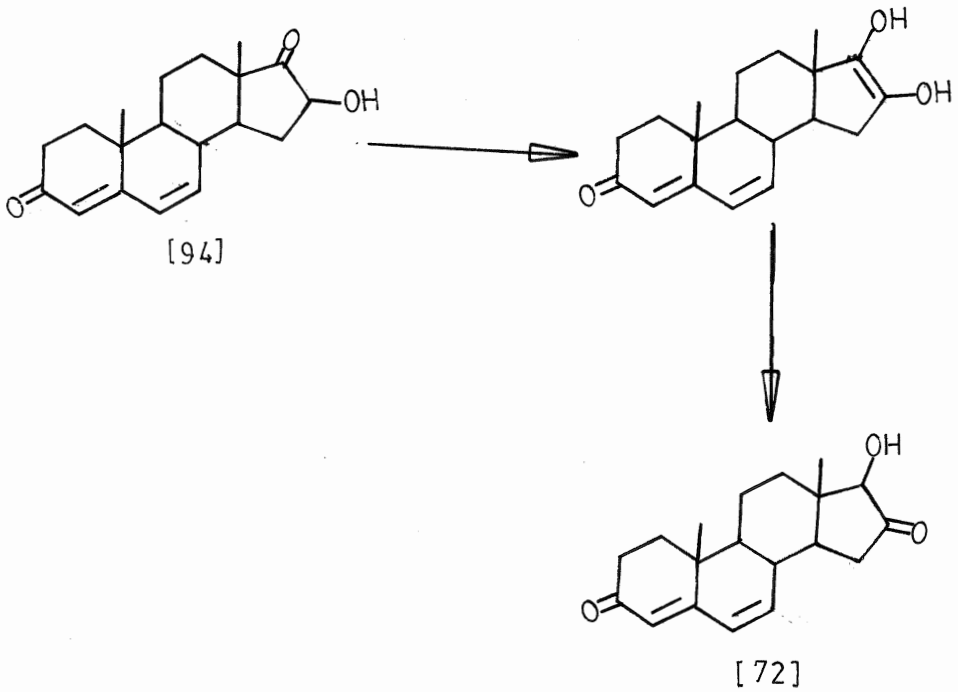


[92]



[60]

Figure 14. Rearrangement of 16β-hydroxyandrost-4,6-diene-3,17-dione to 17β-hydroxyandrost-4,6-diene-3,16-dione



[94]

[72]

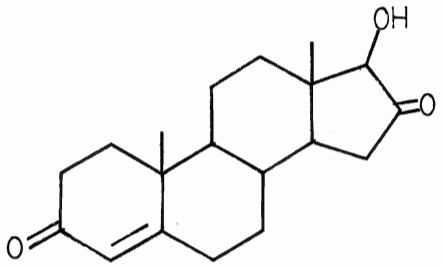
examination of the $^{1}H$ NMR and $^{13}C$ NMR spectra. The $^{13}C$ NMR spectrum was especially informative since it indicated that the hydroxy group was located at C-17 and that the $\Delta^{4,6}$-3-dienone function remained unaffected. That the carbonyl group was α to the 17-hydroxy group was supported by the observation of a positive red tetrazolium test.[106] That the mass spectrum showed a molecular ion at 300 (16 mass units heavier than the substrate) confirmed that [72] was a monohydroxylated metabolite.

There is a precedent for the rearrangement just mentioned above since Dodson and Mizuba[107] isolated 17β-hydroxyandrost-4-ene-3,17-dione [95] from incubation of androst-4-ene-3,17-dione [10] with Corticum centrifugum. In another study [95][108] was also isolated when either [96] or [10] was fermented with A. niger.

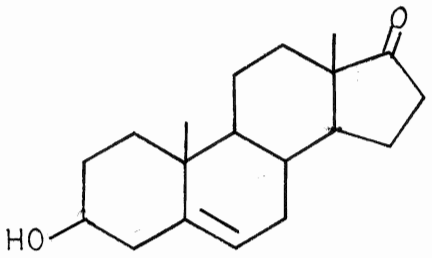
The rearrangement of [94] to [72] is supported by the report that treatment of [97] with dilute sulfuric acid in methanol at room temperature furnished [95].[78]

It is not possible to determine unambiguously whether the 16α or 16β hydrogen is lost on oxidation, but since it was reported that 16β-acetoxy-17-oxo steroids were hydrolysed to the 16-oxo-17-hydroxy steroids whereas 16α-acetoxy-17-oxo steroids gave only 16α-hydroxy-17-oxo steroid, it is assumed that [72] resulted from 16β-hydroxylation of [60].[77,78]

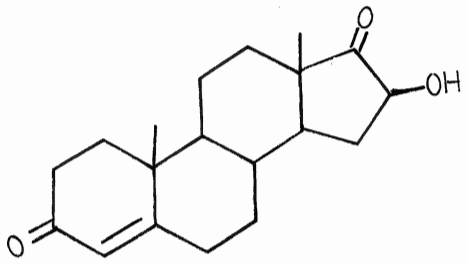
The fermentation of [10] with R. arrhizus furnished 6β- and 11α-monohydroxy products. Therefore it is rather surprising that the introduction of a second double bond would result in the complex mixture




[95]




[96]




[97]

of products obtained in the latter case. While these results are surprising, previous studies[33,45] have shown that the introduction of unsaturation in other substrates also had dramatic effect on the types of products obtained. This phenomenon is well-illustrated by comparison of the products obtained from fermentation of 3β-hydroxypregnan-20-one [98] and 3β-hydroxypregn-16-ene-20-one [99] (see Table 5). The differences here are attributed to the allylic nature of the 15 position than to the restricted rotation of the 17-acetyl group.[33]

The suggestion has been made that the different products obtained before and after introduction of unsaturation could be the result of increased rigidity and distortion of the substrate. In the present study molecular models have shown that the introduction of a double bond at the C6-C7 position of [10] increases the planarity of the AB rings, which may in part account for the epoxidation from both the α- and β-face.

It is therefore apparent that the introduction of unsaturation affects the metabolic disposition of the substrate through as yet obscure and complex means.

In summary then, 21-halogenated and 21-methyl steroids are not metabolized to any significant extent by the fungus Aspergillus niger.

The placement of a fluorine atom at a favoured site of hydroxylation blocks hydroxylation at that site but directs hydroxylation to other positions in the steroid nucleus in the case of Curvularia lunata, whereas the presence of the fluorine in the immediate vicinity of the favoured site of hydroxylation slowed the rate of hydroxylation at that position but did not prevent its occurrence in the case of Rhizopus

Table 5. Comparison of the products obtained before and after introduction of unsaturation into a steroidal substrate[33,45]

| Fungus | 3β-Hydroxypregnan-3,20-dione [98] Products (%) | 3β-Hydroxypregn-16-ene-3,20-dione [99] Products (%) |
|---|---|---|
| Calonectria decora | 11β,15α-dihydroxy-(24)<br>3-CO-17β,12β,15α-trihydroxy(8) | none recovered |
| Daedalea refescens | 7α-Hydroxy-(9)<br>7β-Hydroxy-(9) | 14α,15β-dihydroxy-(21) |
| Rhizopus arrhizus | 7β,12β-dihydroxy-(22)<br>7β,11α-dihydroxy-(10)<br>7β-hydroxy-(8) | 7α-hydroxy-(13)<br>7α,15α-dihydroxy-(12)<br>7-CO-15β-hydroxy-(12) |

stolonifer. Also the presence of an hydroxy group geminal to the favoured site of hydroxylation did not appear to affect the metabolism of that substrate when incubated with R. stolonifer.

The incubation of a substrate unsaturated at a favoured site of hydroxylation with R. arrhizus provided a complex mixture of products. The isolation of both the axial and equatorial epoxide was rationalized not as a violation of the postulation by Bloom and Shull, but as a result of the lack of regio- and stereospecificity of the 6β-hydroxylase enzyme.

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